P
PR
88
1153
no. 2

THE JOURNAL OF PHYSIOLOGY

THE UNIVERSITY OF MICHIGAN
OCT 11 1960
SCIENCE LIBRARY

EDITED FOR

THE PHYSIOLOGICAL SOCIETY

BY

N. AMBACHE
P. M. DANIEL
G. W. HARRIS
R. J. LINDEN
R. V. COXON
S. P. DATTA
R. C. JORDAN
H. R. NOLTIE
M. DE B. DALY
G. GORDON
B. KATZ
W. J. O'CONNOR
W. D. M. PATON
G. S. BRINDLEY
(Secretary)
W. A. H. RUSHTON
R. D. KEYNES
(Chairman)

September 1960
Volume 153, No. 2

CAMBRIDGE UNIVERSITY PRESS
Bentley House, 200 Euston Road, London, N.W. 1
American Branch: 32 East 57th Street, New York 22, N.Y.

Price 30s. net
(U.S.A. $5.00)

PAGE

Asher, H. M. F. An instrument for measuring refractive errors 33P

Machin, K. E. and Pringle, J. W. S. Rhythmic properties of insect fibrillar muscle 34P

Aubert, X. M. J. and Keynes, R. D. Measurement of temperature changes during the discharge of *Electrophorus electricus* 35P

Gray, E. G. and Whittaker, V. P. The isolation of synaptic vesicles from the central nervous system 35P

Glaser, E. M., Griffin, J. P. and Knight, D. Apparatus for recording the heart rate in conscious animals 37P

Cambridge, G. W. and Haines, J. A method of applying temperature compensation to capacitance transducer heads 38P

Reid, C. S. W. Eructation and rumen movements in sheep 39P

Robertson, J. D. Myelinating and non-myelinating nerve fibres during development: a new component of the endoplasmic reticulum of Schwann cells 40P

Bingley, M. S. and Saunders-Singer, E. A micromanipulator for the penetration of Protozoa and biological tissues with saline-filled electrodes 42P

Bingley, M. S. A mains-driven biological DC amplifier 42P

Matthews, B. H. C. A model of auditory ossicles (film) 42P

Glees, P. Axo-dendritic synapses of the tectospinal tract in the chicken . 42P

Donaldson, P. E. K. and Barlow, H. B. Some tricks with correlation . . . 43P

Donaldson, P. E. K. A versatile console for electrophysiology 43P

Howarth, F. Multi-channel pressure recording 43P

Verney, E. B. A technique for reversible functional adrenalectomy in the living dog 43P

Armett, Christine J. and Ritchie, J. M. The effect of anticholinesterases on the action of acetylcholine on mammalian non-myelinated nerve fibres . . 43P

Daniel, P. M., Pratt, O. E. and Treip, C. S. Changes in adrenal cortical function after pituitary stalk section in the rhesus monkey 44P

Marriott, F. H. C. and Morris, Valerie B. The distribution of light in an image formed in the cat's eye. 45P

Lloyd, D. P. C. Temperature and the action of denervated sweat glands in the cat's foot pad 43P

Keatinge, W. R. The parts played by superficial and by deep temperature receptors in raising the metabolic rate of fat and thin men exposed to cold . . . 46P

Sinclair, L. The effects of intraventricular iproniazid on the rat 47P

Eccles, J. C., Kozek, W. and Magni, F. Dorsal root reflexes in muscle afferent fibres 48P

Eccles, Rosamond M., Iggo, A. and Ito, M. The distribution of recurrent inhibition among motoneurones 49P

Waddell, A. Wilma and Weatherall, M. Separation of slowly-exchanging fractions of the potassium of rabbit auricles 50P

Strange, Priscilla H. The spectral sensitivities of the clawed toad, *Xenopus laevis*, on black and white backgrounds 52P

Blair-West, J., Coghlan, J., Denton, D. A., Goding, J. R., Munro, J. A. and Wright, R. D. The effect of neuraxial ablations upon the secretion of electrolyte-active steroid 52P

Tregear, R. T. Epidermis as a thick membrane 54P

Holzbauer, Margarethe and Vogt, Marthe. The effect of anoxia produced by removal of red cells on aldosterone secretion from the dog adrenal 55P

McLennan, H. Evidence for the presence of an inhibitory transmitter substance in Factor I extracts 55P

Mills, J. N., Thomas, S. and Williamson, K. S. The different effects of aldosterone and cortisol in recumbency 56P

GE
P
P
P

J. Physiol. (1960), **153**, *pp.* 209–217
With 7 text-figures
Printed in Great Britain

ELECTRICAL AND MECHANICAL FACTORS IN THE ADAPTATION OF A MAMMALIAN MUSCLE SPINDLE

BY O. C. J. LIPPOLD, J. G. NICHOLLS* AND J. W. T. REDFEARN†
From the Department of Physiology, University College London

(*Received* 7 *August* 1959)

Sensory nerve endings usually respond to maintained stimulation by the generation of a train of impulses declining in frequency with time. In some receptors, for instance the Pacinian corpuscle (Hubbard, 1958) and receptors in the skin of the frog (Loewenstein, 1956), adaptation largely depends upon mechanical factors. In these instances adaptation is rapid, and the impulse frequency falls abruptly to zero; the muscle spindle behaves differently in that its response consists of two phases—an initial high-frequency burst, followed by a steady discharge (Matthews, 1931, 1933; Katz, 1950; cf. Eyzaguirre & Kuffler 1955).

It seemed likely that the rapid first phase in the discharge of the muscle spindle was analogous to the quickly adapting response of the Pacinian corpuscle, and was therefore also due to mechanical factors. If the electrical properties of the membrane of the nerve terminals were responsible for the adaptation, then one would expect that electrical polarization applied to the nerve terminals themselves would also produce such an adaptation in two phases. If polarization does not give the two phases which are produced by stretch, then the difference might be due to mechanical adaptation.

We have recorded from a single mammalian muscle spindle and have compared the effects of electrical and mechanical stimulation on the same ending.

METHODS

Preparation. In all experiments the tenuissimus muscle was dissected from a cat or kitten previously anaesthetized with sodium pentobarbital 40 mg/kg (Abbott Laboratories) injected intraperitoneally. We used the central part of the muscle, which contains 5–10 stretch receptors arranged in series longitudinally down the middle (Boyd, 1956). The isolated preparation was mounted in Krebs's solution of composition (mM): NaCl 115, KCl 4·6, $NaHCO_3$ 24·1, $CaCl_2$ 2·46, $MgSO_4$ 1·15, KH_2PO_4 1·15, glucose 8·85. A mixture of 95% O_2 and 5% CO_2 was bubbled through the bath. The bath contained 15–20 ml. Krebs's solution, which was changed at frequent intervals. The temperature could be maintained at any desired level, usually 37° C. The muscle was clamped at one end by a pair of forceps (insulated

*Beit Memorial Fellow. Present address: University Laboratory of Physiology, Oxford.
† Present address: The Medical Research Council's Clinical Psychiatry Research Group, Graylingwell Hospital, Chichester.

from earth) on a rack and pinion so that the resting length could be varied. The other end of the muscle was tied to a rod through which it could be given step-wise or sinusoidal stretches. The square-wave mechanical displacement was produced by an electromagnet or a vacuum-operated bellows. The sinusoidal displacement, of variable frequency or amplitude, was applied by means of a cam and slider system. The resulting movement of the muscle was displayed on one beam of an oscilloscope by means of a transducer (RCA 5734).

Recording. The recording of afferent activity was made from single spindles. Single afferents were obtained at first by the laborious process of splitting down the main nerve trunk to the muscle. A far more convenient method was to make use of the anatomical features of the preparation, which are shown in Fig. 1. The small twigs usually contained one or two afferent fibres from muscle spindles, in addition to motor fibres. Single-fibre preparations were obtained by dividing these twigs. Responses from single spindles could be recorded up to 36 hr after the dissection. The preparation would also survive for many hours immersed in liquid paraffin.

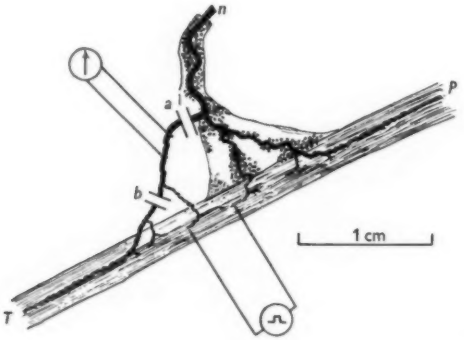


Fig. 1. Diagram showing tenuissimus muscle of the kitten with nerve supply (*n*) and position of recording and stimulating electrodes for polarization experiments. *P* is the pelvic end and *T* the tibial end. Cuts have been made at (*a*) and (*b*) leaving only the twig for recording.

The nerve was placed on the wick of a non-polarizable electrode which consisted of an Ag–AgCl wire dipping into an agar-saline bridge. Electrodes were arranged either for dipolar recording from the nerve, or with one electrode on the muscle (Katz, 1950). The nerve, and in some cases the whole preparation, could be raised into a layer of liquid paraffin for recording purposes. A cathode-follower input stage fed into either an a.c. or a d.c. amplifier connected to a cathode-ray oscilloscope. Grid current was less than 10^{-11} A. Polarizing electrodes were of the same form as the recording electrodes and were placed on the muscle on each side of the supposed position of the nerve ending; in some cases one electrode was put on the nerve twig itself (Edwards, 1955).

Square waves were produced by a circuit giving pulses of variable duration, polarity and voltage, balanced about earth; a swamping resistance of 4·7 MΩ in series with the preparation was used to eliminate the effects of changes in resistance of the preparation.

It was necessary to ensure that the applied current was polarizing the nerve terminals and not producing its effects by direct stimulation of the nerve trunk or the muscle. The following precautions were taken: the muscle was raised into paraffin and blotted to remove saline and so prevent current spread; it was carefully observed under the microscope so that current strengths could be kept well below those which caused twitching of individual

fibres; curare 5×10^{-6} (w/v) was used as an additional safeguard, although with the stimulus strengths used there was no evidence that large or small motor fibres were being excited. Only if considerably stronger stimuli were used did spikes from other fibres appear in the recording. The criteria for assuming that the spikes produced by the polarizing current were due to an effect on the ending, rather than on the whole nerve trunk, were (*a*) the effects of the current and stretch of the muscle would summate, (*b*) pinching the nerve within the muscle as close to the receptor as possible abolished the effect, (*c*) the position of the electrodes was critical to within 1 mm, (*d*) the effect was not obtained by simply stimulating the nerve, and (*e*) when current flowing in one direction increased the rate of firing, then reversing the flow decreased it.

RESULTS

Polarization of the nerve endings

In a slightly stretched muscle, showing a discharge from the spindle, the application of a direct current through a cathode near the receptor increased the frequency of firing. If the muscle was relaxed, and there was no discharge, then a train of impulses was initiated. If the polarity was reversed with the anode near the receptor, the frequency was reduced or firing was abolished (Fig. 2).

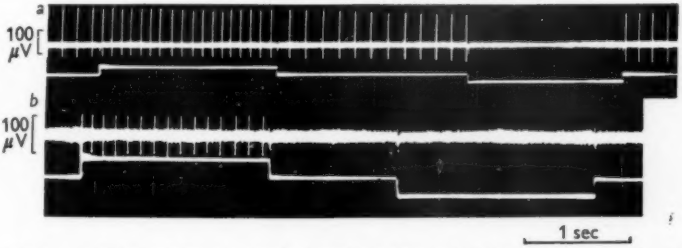


Fig. 2. *a*, Effect of d.c. on steady discharge due to stretch. Upper record, action potentials from single muscle spindle; lower record, current flowing through preparation, upwards spindle −ve, downwards spindle +ve. Depolarization causes increase in frequency; hyperpolarization stops the discharge. *b*, Effect of d.c. on relaxed spindle. Only depolarization causes a discharge.

The alteration in frequency was closely related to the applied d.c. (Fig. 3), as in the frog (Edwards, 1955). The relationship did not hold when the d.c. produced twitching or stopped the discharge completely.

The discharge produced by d.c. was regular, and the frequency remained constant during the entire period of depolarization (Figs. 2 and 4). In Fig. 4 it can be seen that the frequency was 27/sec when the d.c. was applied and was still the same after 1·3 sec. At threshold, d.c. either initiated a regular train of impulses or none at all. In a few spindles there was a gradual decline in frequency of a few impulses per second during the course of polarization, but this never amounted to more than 10 % of the initial frequency. There was an upper limit to the frequency which could be

obtained by depolarization, because if the voltage was too high stimulation of the muscle fibres interfered by producing a contraction. If the muscle was observed to twitch with these high voltages a high-frequency burst of firing was often seen, presumably as a result of intrafusal contraction. Only voltages well below twitch threshold were used in our experiments.

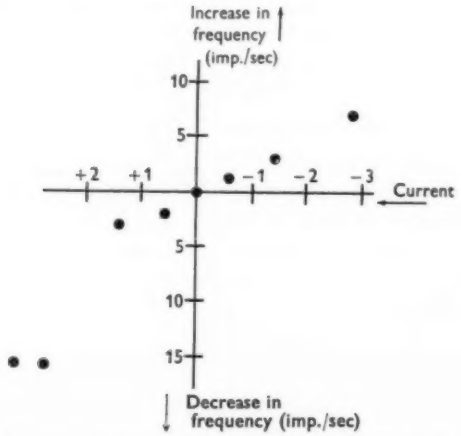


Fig. 3. The relationship between change in firing frequency (ordinate) and applied d.c. in relative units. Current flow through muscle less than 4 μA. Muscle slightly stretched to give a steady discharge of 16/sec, hence points corresponding to −16/sec indicate that polarizing current has stopped the discharge.

The effect of stretch

At 37° C the spindles were silent when the muscle was relaxed. The effect of mechanical stretch was quite different from that of d.c. The response occurred in two phases, an initial burst of impulses at a high frequency followed by a maintained discharge at a much lower frequency (Matthews, 1931, 1933; Katz, 1950).

In Figs. 4 and 5 the effects of stretch and depolarization on the same muscle spindle are shown. With the stretch response the firing frequency in the initial burst was 92/sec and fell to 25/sec in 0·3 sec. It remained at about 25/sec for a further 1 sec. This is in contrast with the constant frequency of 27/sec maintained during the entire period of electrical stimulation. This difference was found in all twenty fibres.

In contrast again with the effect of d.c. on the endings, a threshold stretch produced only the first phase. If the initial tension on the muscle was increased, the same increment of stretch now gave both phases. A tension between these gave the first burst, followed by only a few impulses of the second phase (Fig. 6). If the same experiment was done using d.c.

instead of added stretch (i.e. initial length was altered and a given d.c. pulse was used each time) the spindle responded with a train of impulses at constant frequency or not at all.

Spindles damaged, either during dissection or deliberately, had quite different responses. The threshold was higher, firing was irregular, sometimes in rhythmical bursts and often of high frequency. All such fibres were rejected.

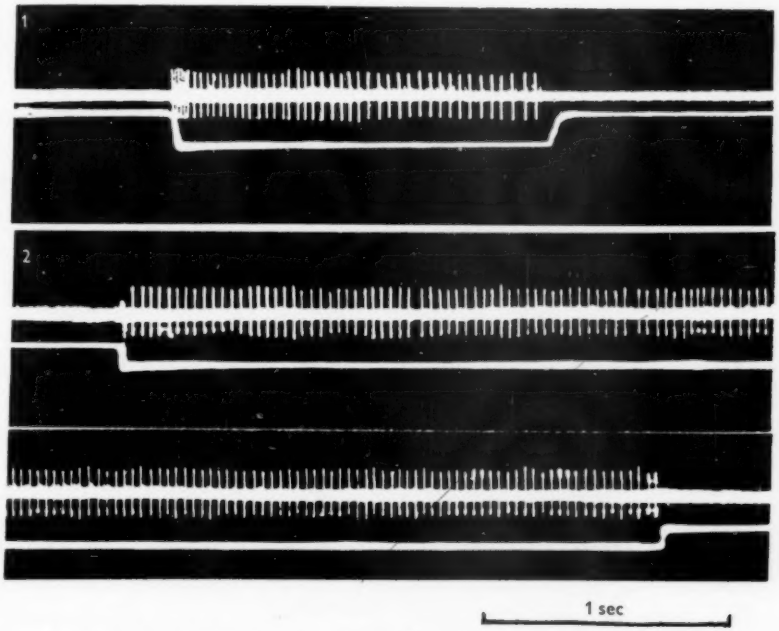


Fig. 4. The effect of stretch (1) and d.c. (2) on the same muscle spindle. In (1) lower beam records displacement; there is a high-frequency burst initially, followed by a steady discharge. In (2) depolarization (lower beam) gives rise only to a maintained discharge. The bottom records are the continuation of the middle ones.

The receptor potential

The receptor potential recorded during mechanical stretch, after procaine (0·01 %), also showed two phases. It was largest during the initial stretch and then declined (Fig. 7). The frequency of firing was proportional to the magnitude of the receptor potentials. On relaxation of the muscle after a stretch there was frequently an 'off effect' which consisted of a brief hyperpolarization. This was previously found in the frog (Katz, 1950).

These receptor potentials were distinguished from movement artifacts

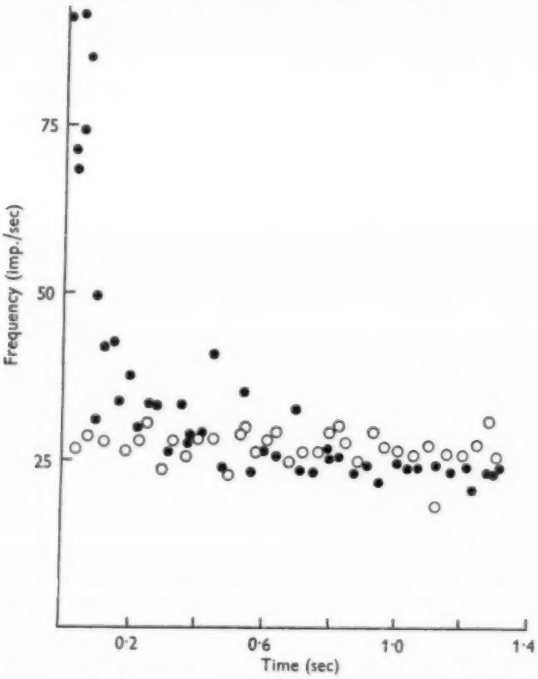


Fig. 5. Relationship between frequency of firing and time after stretch (●) and d.c. (○) applied at time 0 to a single spindle.

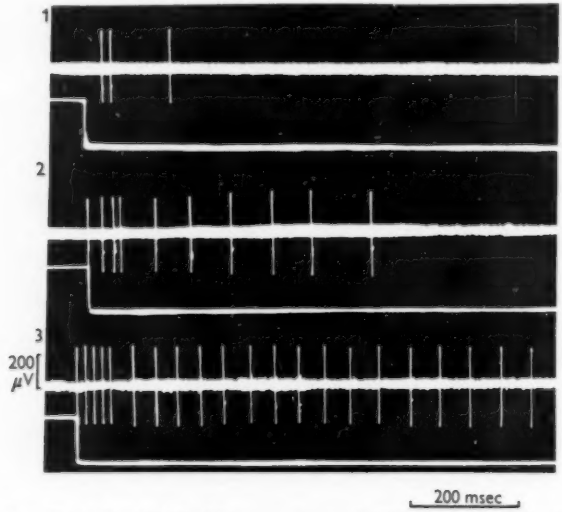


Fig. 6. The effect of a stretch of 1 mm on a muscle (1) completely relaxed, (2) held at 5 % greater length, (3) at 10 % greater length.

in the usual way. If the nerve was placed in contact with muscle surface, then electrotonic spread was short-circuited, and the receptor potentials disappeared. Pinching the nerve had a similar effect. Furthermore, if the recording interface was near enough to the ending, changes in the shape of the action potential could be observed even without procaine. The action potential was of longer duration and was preceded by a gradually increasing depolarization (Lippold, Nicholls & Redfearn, 1960).

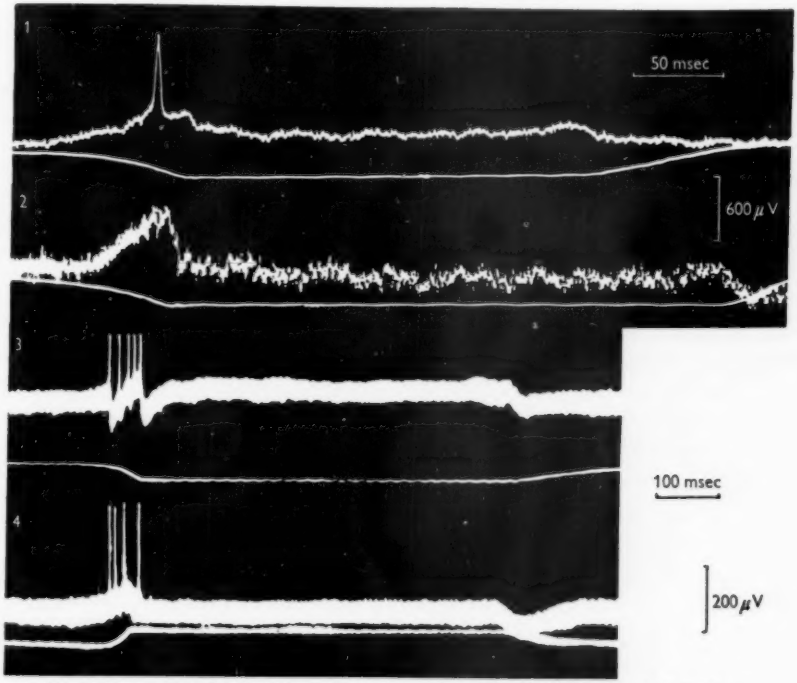


Fig. 7. Receptor potentials (upper beam) recorded from two different spindles when stretched (lower beam): (1) shortly after procaine 0·01 % showing one spike followed by decline in receptor potential; (2) 15 min after procaine, pure receptor potential (gain increased 3×); (3) and (4) another fibre shortly after procaine showing receptor potential and 'off effect'. In (4) stretch is signified by an upward deflexion of the lower beam.

DISCUSSION

When a muscle is stretched, its muscle spindles give a discharge initially of high frequency (the 'dynamic phase'), the peak value of which depends upon the rapidity of the stretch; subsequently the discharge rate falls to a lower frequency (the 'static' phase) which is related to the degree of extension of the muscle. This decline in the discharge rate during a

maintained stretch is the adaptation of the sensory mechanism. Theoretically it could be due entirely to the physical properties of the linkage between the sensitive nerve terminal and the tendon which is being stretched. It could also be due to the behaviour of the nerve membrane, which might introduce a differential component in its electrical response to an undistorted mechanical input.

On the basis of previous work in the frog it might have been predicted that both mechanical and electrical factors were operative in the process of adaptation. First, Katz (1950) showed that the receptor potential had an overshoot during the dynamic phase of a rapid stretch. Secondly, Matthews (1931) found that the discharge from spindles in the frog's toe muscle declined with time when the nerves were artificially depolarized by the passage of a constant current through them. However, the frog stretch receptor differs greatly from that in the mammal both in structure and in function, and further experimental evidence is needed to make a clear distinction between the operation of the two factors.

Our experiments indicate that adaptation in the mammalian spindle occurs as a result of the physical characteristics of the spindle and extrafusal muscle. When a rectangular mechanical pulse is used to stretch the muscle, the resultant receptor potential is distorted. It is not rectangular but has a 'hump' which coincides with the instant of application of stretch and the magnitude of this hump varies with the rate of stretching. This is the effect to be expected if the terminal membrane were sensitive to the mechanical stress within the muscle and if this stress were proportional to the first differential of the applied strain.

From rough estimates in experiments such as that of Fig. 7 it does appear that no additional adaptation is introduced by a non-linearity in the relation between firing frequency and receptor potential during the dynamic phase of the stretch (cf. Katz, 1950). We have verified this directly by producing a depolarization of the terminal with an applied current. A rectangular current pulse initiates a non-adapting train of impulses, whose frequency is roughly proportional to the current. There is never an initial burst of impulses at a high frequency when the polarizing current is first applied. Matthews (1931) described, in the toe muscles of the intact frog, a certain amount of electrical adaptation to heavy polarizing currents, but it is fairly clear that he was forced to use high current densities in his muscles, which led to difficulties with electrode polarization and contraction of intrafusal and perhaps extrafusal muscle fibres. Edwards (1955), on the other hand, did record in isolated frog muscle a non-adapting discharge from the muscle spindles when he put a voltage across them.

This comparison between the response to mechanical stretch, which produces 'overshoot' in the receptor potential (and thus the initial dynamic

phase in the discharge), and that due to polarization of the nerve endings, which gives rise to an unvarying discharge, is clear evidence that no adaptation occurs in the membrane itself. It is therefore the result of physical processes involved in the transmission of forces from the tendon to the nerve ending. Adaptation can be pictured as a physical deformation in the region of the terminals which is exaggerated when a muscle is stretched rapidly, followed by restitution to a lower or 'adapted' level determined by the degree of stretch.

A further possibility is that an undistorted mechanical displacement is transmitted to the nerve terminal and that the conversion of this to a depolarization is non-linear. Nothing is known about this transducer system and it does not seem profitable to speculate about its possible role at present.

SUMMARY

1. The adaptation of muscle spindles has been studied in the isolated tenuissimus muscle of the kitten.

2. The effects of mechanical stretch and of depolarization with applied direct current have been compared in the same single muscle spindle. There is no adaptation to direct current but there is a pronounced initial fall in frequency of the discharge due to stretch, which corresponds to a decline in the receptor potential.

3. On the basis of this difference it is concluded that adaptation in the mammalian muscle spindle is mechanical in origin or occurs during the generation of the receptor potential.

We are indebted to the Nuffield Foundation for a grant in support of this work.

REFERENCES

Boyd, I. A. (1956). The tenuissimus muscle of the cat. *J. Physiol.* **133**, 35–36*P*.

Edwards, C. (1955). Changes in the discharge from a muscle spindle produced by electrotonus in the sensory nerve. *J. Physiol.* **127**, 636–640.

Eyzaguirre, C. & Kuffler, S. W. (1955). Processes of excitation in the dendrites and in the soma of single isolated sensory nerve cells of the lobster and crayfish. *J. gen. Physiol.* **39**, 87–120.

Hubbard, S. J. (1958). A study of rapid mechanical events in a mechano-receptor. *J. Physiol.* **141**, 198–218.

Katz, B. (1950). Depolarization of sensory terminals and the initiation of impulses in the muscle spindle. *J. Physiol.* **111**, 261–282.

Lippold, O. C. J., Nicholls, J. G. & Redfearn, J. W. T. (1960). A study of the afferent discharge produced by cooling a mammalian muscle spindle. *J. Physiol.* **153**, 218–231.

Lippold, O. C. J., Redfearn, J. W. T. & Vučo, J. (1958). The effect of sinusoidal stretching upon the activity of stretch receptors in voluntary muscle and their reflex responses. *J. Physiol.* **144**, 373–386.

Loewenstein, W. R. (1956). Excitation and changes in adaptation by stretch of mechanoreceptors. *J. Physiol.* **133**, 588–602.

Matthews, B. H. C. (1931). The response of a single end organ. *J. Physiol.* **71**, 64–110.

Matthews, B. H. C. (1933). Nerve endings in mammalian muscle. *J. Physiol.* **78**, 1–53.

J. Physiol. (1960), **153**, *pp.* 218–231
With 13 *text-figures*
Printed in Great Britain

A STUDY OF THE AFFERENT DISCHARGE PRODUCED BY COOLING A MAMMALIAN MUSCLE SPINDLE

By O. C. J. LIPPOLD, J. G. NICHOLLS* and J. W. T. REDFEARN†
From the Department of Physiology, University College London

(*Received* 7 *August* 1959)

During experiments on muscle spindles we found that if the temperature of an isolated mammalian muscle fell a few degrees below body temperature an afferent discharge suddenly appeared. This response resembles that of a cold receptor (Hensel & Zotterman, 1951*a*). Other receptors respond to cooling although this is not their primary function, e.g. pressure receptors (Dodt & Zotterman, 1952) and the lateral-line organ of the Japanese sea-eel (Katsuki, Yoshimo & Chen, 1950).

With an isolated muscle it is possible to alter the ionic environment, to apply stretch and to polarize the ending electrically. We have used these procedures to study the mechanism by which cooling produces afferent nerve impulses.

METHODS

In general the experimental techniques were those described in the preceding paper (Lippold, Nicholls & Redfearn, 1960).

Preparation. The isolated tenuissimus muscle of the cat or kitten was used in a temperature-controlled bath containing 15 ml. of Krebs's solution (mM: NaCl 115, KCl 4·6, $NaHCO_3$ 24·1, $CaCl_2$ 2·46, $MgSO_4$ 1·15, KH_2PO_4 1·15, glucose 8·85); 95 % O_2 and 5 % CO_2 was bubbled through it. Temperature was measured by a thermometer of low thermal capacity or a thermocouple next to the muscle. The temperature of the bath could be altered at rates up to 0·2° C/sec by changing the temperature of water flowing through an outer jacket. Recordings were made from single muscle spindle afferents with the nerve on non-polarizable electrodes in a layer of liquid paraffin.

Whenever the ionic concentration of the Krebs's solution was to be altered, kitten muscles were used. In these thin muscles diffusion is rapid and there is no evidence that the spindles behave differently from those in the adult cat.

Electronically generated square pulses served either to polarize the nerve terminals or to stimulate the muscle for the purpose of identifying the type of endings. The action potentials were photographed on moving film; in some experiments, since the discharge was regular, it was possible to measure frequency by counting the number of spikes during a 10 sec period by electronic methods.

Experiments in vivo. Experiments were also carried out on muscles in the anaesthetized cat; either the whole animal or the limb alone was cooled. Recordings were made from

* Beit Memorial Fellow. Present address: University Laboratory of Physiology, Oxford.
† Present address: The M.R.C.'s Clinical Psychiatry Research Group, Graylingwell Hospital, Chichester, Sussex.

single dorsal-root afferent fibres, the ventral roots having been cut previously (Lippold, Redfearn & Vučo, 1958). The tendon of tibialis anterior or gastrocnemius was freed completely from its insertion but the skin over the muscle was not disturbed. The temperature of the muscle and the rectal temperature were measured with thermometers.

Conduction velocity was measured by using three electrodes, separated by known distances and connected to separate amplifying channels. Measurements were made from the rising phase of action potentials initiated by stretch.

Receptor potentials were recorded from kitten muscle by the method of Katz (1950), using the same criteria for distinguishing between receptor potentials and movement artifacts.

RESULTS

Isolated muscle

When activity from the whole nerve trunk of the isolated tenuissimus was recorded at 37° C, action potentials were present only when the muscle was stretched. There was no activity when the muscle was relaxed. On cooling to about 32° C a single-fibre discharge appeared, followed by others as cooling proceeded, until about six or seven were firing independently. If the muscle was now stretched, the frequency of these potentials increased, and in addition two or three larger spikes appeared. The activity remained at approximately the same frequency while the temperature was lowered to about 28° C, below which there was a gradual decline in frequency.

The characteristics of the response to cold were studied in single afferent fibres from muscle spindles. When the muscle was cooled slowly (less than 1° C/min) the discharge began abruptly at about 32° C. Its frequency was at first approximately 10/sec, gradually rose to a maximum of 15/sec at 28° C, and then declined (Fig. 1). It fell to zero at about 15° C.

At any one temperature the frequency of impulses was constant, and so regular that the action potentials could be synchronized for several minutes with the time base on the oscilloscope. Slow rewarming produced the same frequencies at given temperatures and the discharge stopped again at the temperature at which it had begun.

Rapid changes in temperature caused slightly higher rates of firing, but the final rate depended only on the final temperature and not on the rapidity with which it was reached. When the preparation was rapidly rewarmed from 20° C (1° C every 10 sec), although the frequency was higher throughout the temperature range, the temperature at which the discharge stopped was the same as before (Fig. 2).

In all the experiments it was possible to record the normal response to stretch of the muscle; when the regular cold response was present, stretching produced a sharp increase in rate followed by adaptation (Fig. 3).

On relaxation there was a pause of one or two impulses before the cold response was resumed. This occurred during the phase of hyperpolarization

('off-response') of the receptor potential (Fig. 3) (Lippold *et al.* 1960). It was clear that these were afferents from muscle spindles, since a twitch elicited by direct stimulation of the muscle interrupted the firing due to stretch. In some preparations stronger stimulation produced a burst of impulses presumably due to intrafusal contraction (Fig. 4) (Hunt & Kuffler, 1951*a*, *b*; Matthews, 1933).

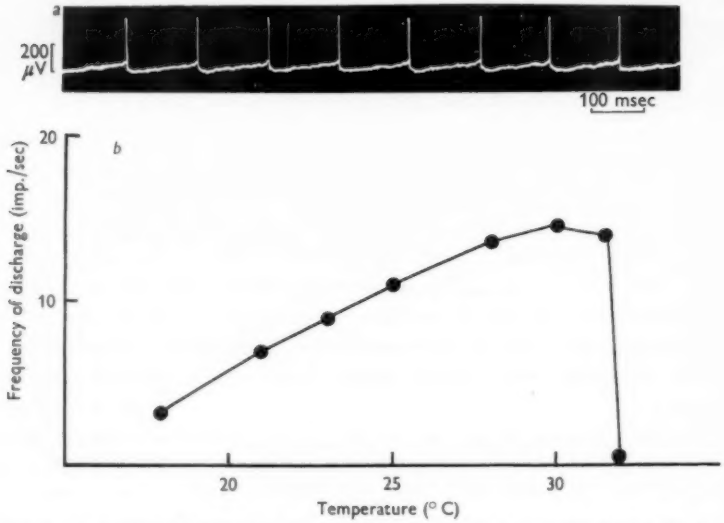


Fig. 1. (*a*) Record of the discharge at 30° C in a single muscle spindle. The electrode was close to the nerve ending and receptor potentials precede each spike. (*b*) The relationship between the frequency of discharge and temperature.

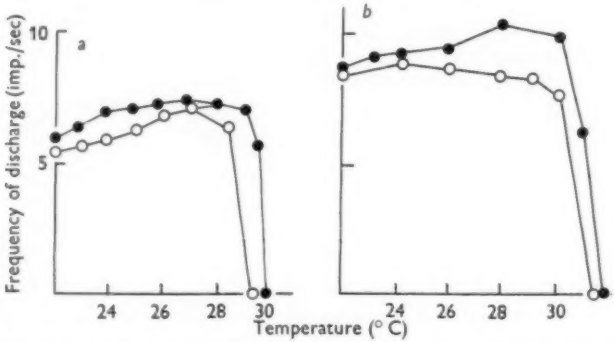


Fig. 2. The relationship between frequency of discharge and temperature, (*a*) during slow cooling and rewarming (1° C/min) and (*b*) during rapid cooling and rapid rewarming (1° C/10 sec). ○, cooling; ●, rewarming.

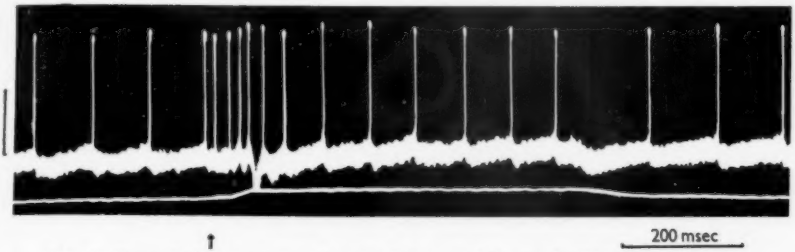


Fig. 3. Record of the response to stretch in a cooled muscle (28° C), upper trace; in lower trace stretch is upwards. With the muscle relaxed there is a steady discharge; stretch gives rise to an increase in frequency followed by adaptation. There is a small receptor potential visible before each spike and a transient hyperpolarization ('off-effect') on release. Onset of stretch at arrow. Voltage calibration 200 μV.

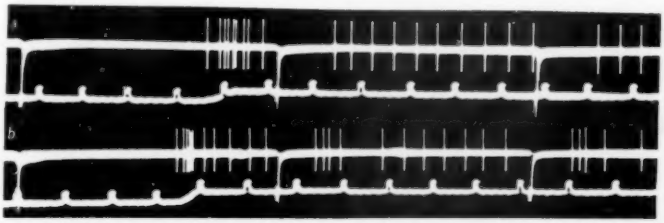


Fig. 4. The effect of electrical stimulation of the whole muscle on the discharge of a spindle when it is stretched (lower beam). (*a*) Weak stimulus (downward stimulus artifact) produces twitch of extrafusal fibres and a silent period in the discharge due to stretch. (*b*) A stronger stimulus introduces a burst in the silent period due to contraction of intrafusal fibres. In both cases the stimulus produces no discharge in the relaxed muscle. Time trace 100 msec.

Experiments on undissected muscle with intact circulation

The effect of cold on spindle discharge was demonstrated in the anaesthetized cat. After the appropriate ventral roots had been cut, there was no activity from relaxed muscle spindles at 37° C. On cooling the whole cat, or the leg alone, the discharge appeared at about 32° C, with the typical frequency of approximately 10/sec (Fig. 5). On rewarming the animal the firing stopped. Stretching the muscle produced a slowly-adapting discharge.

Large and small afferent fibres from tenuissimus

Not all muscle spindles in the isolated tenuissimus became active when cooled. Usually a third of the muscle afferent fibres from which we recorded were inactive unless stretched. This estimate was formed during

the dissection of 70 muscles, while the nerves were being divided. It was confirmed in five muscles in which all afferent fibres were examined; 1 in 3 afferents were found not to fire when cooled although they responded normally to stretch.

The afferent fibres from the endings which did not respond to cold invariably gave the largest action potentials. These spikes were $1\frac{1}{2}$–3 times larger than those conducted from cold-sensitive endings. This was true not only in the dissected single afferents but also in the whole nerve trunk, where it was reasonable to suppose that spike size was proportional to fibre diameter. This was confirmed in an experiment in which the conduction velocities of large and small spikes were measured in a nerve

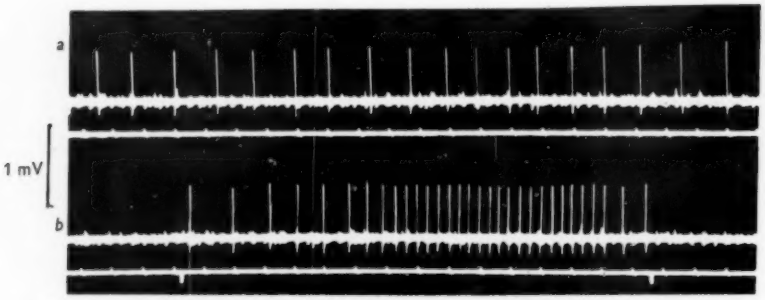


Fig. 5. Cat, 4·2 kg anaesthetized with sodium pentobarbital 40 mg/kg intraperitoneal. Action potentials recorded from single dorsal root filament from gastrocnemius. Ventral roots cut. (*a*) Whole animal cooled, rectal temperature 32° C, muscle completely relaxed. There is a regular discharge. (*b*) Rectal temperature 38° C. Muscle relaxed, no action potentials until, at first downward mark on lower trace, muscle gradually stretched. At second downward mark, muscle released. Time trace 100 msec.

trunk. The spike height was directly proportional to conduction velocity and only those fibres with the largest spikes and the greatest conduction velocity showed no response to cold. Since conduction velocity is proportional to fibre diameter, it follows that there is a cold response only in sensory endings connected to the afferent fibres of smaller diameter. There were also fibres giving spikes of intermediate size which would fire only when the temperature was changed rapidly, although they responded to stretching.

Apart from the spike size and the conduction velocity in the afferent fibre, no difference was found between the nerve endings with and without a cold response.

A few fibres, giving small spikes, have been found to respond to warming as well as to cooling. These fibres began to fire at about 39° C and stopped

when the temperature reached 46° C. Their response to cold and to stretch was the same as in other fibres of similar size (cf. Dodt & Zotterman, 1952).

Mechanical factors

The regular discharge at low temperatures was never affected by curare $5{\cdot}0 \times 10^{-6}$ (w/v), a concentration sufficient to cause complete neuromuscular block. With this concentration, stimulation of the nerve resulted in neither an accelerated discharge nor a silent period (cf. Buchtal & Jahn, 1957).

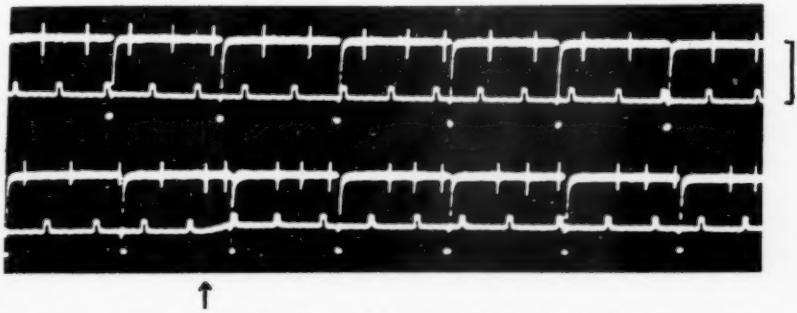


Fig. 6. Effect of direct electrical stimulation of the muscle (dots), upper record during cold discharge, lower record during cold discharge when stretch is applied at arrow (upward movement of lower beam). Stimulation produced a silent period in the stretch response (after arrow) but it had no effect on the cold discharge. Stimulus strength same throughout. Temperature 27° C. Voltage calibration 200 μV. Upper beam, action potentials from single fibre and stimulus artifacts; lower beam, time trace (100 msec).

The discharge was not slowed when the muscle was relaxed as completely as possible; to reduce tension on the spindle to a minimum the muscle, free at both ends, was allowed to rest on the bottom of the bath so that its weight did not stretch it. Even attempts to shorten the spindle by manipulation with needles did not slow the discharge.

A muscle twitch elicited by direct stimulation and contracting the extrafusal fibres was also used to shorten the spindle (Fig. 6). In the completely relaxed muscle this was without effect on the firing rate, which suggested that the cold response was not due to a contracture in series with the spindle. Slightly stretching the muscle caused an increase in frequency and this increase was abolished during the twitch (Fig. 6).

Potassium

There is a quantitative relationship between K^+ concentration and membrane potential (Adrian, 1956), and Katz (1950) has shown that the frequency of firing is proportional to the depolarization at the nerve

terminal. Consequently, a reduction in K^+ which would increase the resting potential should decrease the rate of firing in the cold if it were due to depolarization. The potassium concentration of the Krebs's solution was therefore altered in order to cause changes in the resting potential.

After about 40 min in K^+-free Krebs's solution, the firing began at a lower temperature than usual, and although the temperature–frequency curve was of the characteristic shape, the frequency throughout was reduced. The response to stretch was still present. Increased K^+ concentration raised the rate of firing and extended the temperature range over which it occurred. The concentration of K^+ used was insufficient to stimu-

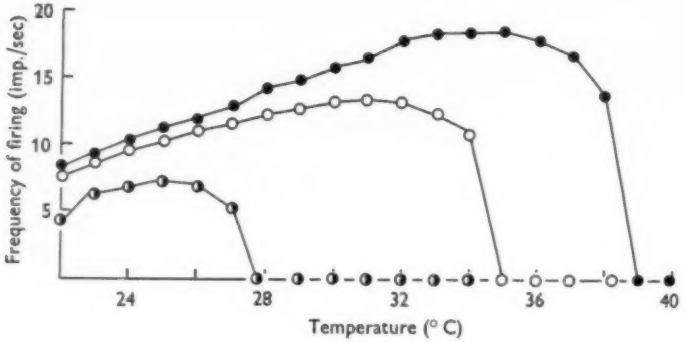


Fig. 7. Frequency of firing plotted against temperature (abscissa) of a single spindle in different K^+ concentrations: ●, 8·6 mM K^+; ○, 5·75 mM K^+; ◑, K^+-free Krebs's solution.

late the nerves directly, to produce muscle contraction, or to interfere with the stretch response. If the nerve was pinched within the muscle all firing was abolished. The effects of both increased and decreased K^+ concentration were reversible on soaking the muscle in normal Krebs's solution for 30–45 min (Fig. 7).

We have shown previously that the responses to cold were found in endings innervated by fibres of small diameter. When using high K^+ concentrations we often observed characteristic cold discharges in the larger fibres which normally were silent unless stretched.

Polarization of nerve terminals

An increase in frequency of the discharge due to cold was brought about by depolarization of the nerve terminals with direct current. Conversely a decrease or cessation occurred when the current was reversed and the terminal hyperpolarized. This alteration in frequency was directly

proportional to the current flow (Fig. 8). Stringent precautions (cf. Lippold *et al.* 1960) were taken to ensure that the nerve terminals alone were being excited.

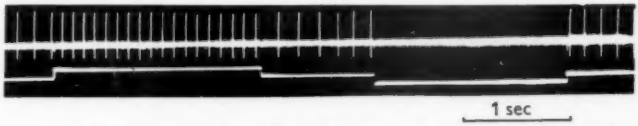


Fig. 8. Polarization of nerve terminals. The lower trace is the current flowing through the preparation; upward deflexion, spindle −ve. The cold discharge (upper trace) is accelerated by depolarizing the spindle and stopped by hyperpolarizing it.

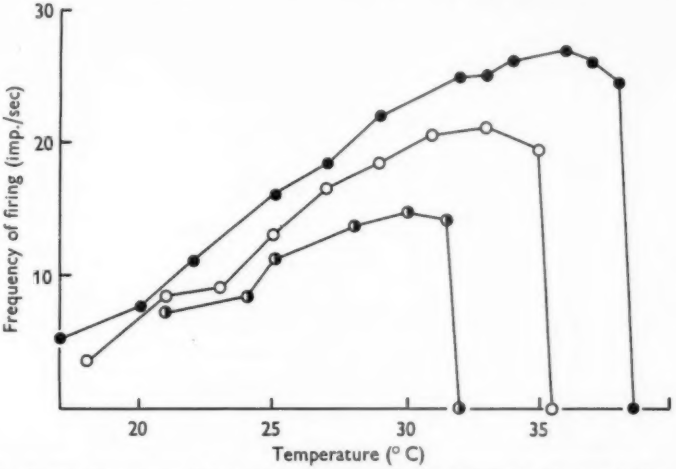


Fig. 9. The frequency of firing plotted against temperature of a single spindle at different muscle lengths, where ◑ is at 95 % of the resting length in the body, ○ is at 110 % and ● is at 131 %.

Alteration in resting length

Another way of depolarizing the nerve terminals is to keep the muscle slightly stretched but not sufficiently to evoke a maintained stretch response. This produced the same changes in firing-rate and temperature–frequency curves as did increased K^+ concentrations (Fig. 9). In all these procedures, pinching the nerve at its entry to the muscle abolished impulses due to cold, stretch and polarization.

Measurements of threshold

The threshold to stretch is reduced at the lower temperatures. We measured the threshold at different temperatures in two ways: one involved a step-wise stretch of the muscle, the other a sinusoidal displace-

ment of varying magnitude but of fixed frequency (Lippold *et al.* 1960). In each case threshold was defined as the minimum displacement producing a single action potential. These determinations of threshold were carried out on fibres of large diameter which did not fire as a result of cooling, because a cold response masked threshold effects. The threshold in both types of experiment progressively fell as the temperature was lowered and reached a minimum value at about 30° C (Fig. 10), which is about the temperature at which the cold response is greatest.

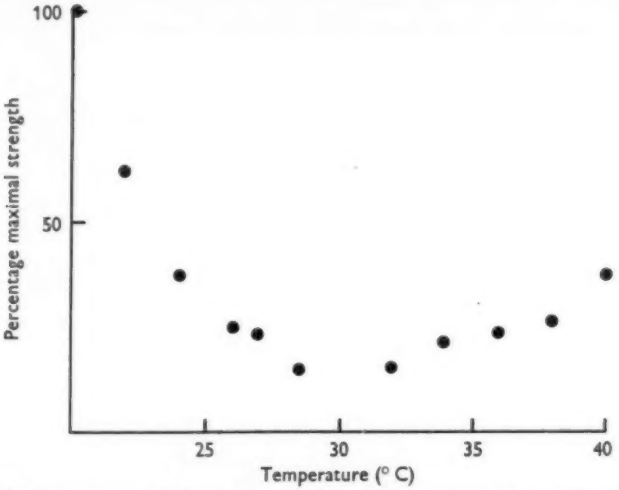


Fig. 10. Measurements of threshold to stretch at different temperatures. Threshold is measured with sinusoidal displacements in a single spindle which had no cold response (see text). Ordinate, % maximum stretch.

The above experiments were designed to measure the threshold of stimulation for a single spike; it is also possible to measure a different threshold—the minimum stretch required for a discharge lasting 10 sec or more. This threshold fell as the temperature was lowered and reached a minimum at 26° C.

A similar relation between threshold and temperature was found in fibres which did have a cold response. In this case it was necessary to measure the minimum stretch required to produce an increase in frequency. The threshold declined as the temperature fell from 37° C to about 28° C, and then rose steeply as the temperature was lowered still further, although the cold discharge was present. Quantitative measurement was difficult, but there was no obvious difference between the large and the small fibres in this respect.

The sensitivity of muscle spindles to a given stretch was measured at different temperatures in most experiments. A stretch at 37° C, which would evoke a high-frequency burst of impulses (300/sec), would produce at 20° C a just detectable increase in frequency of the firing due to the cold discharge. This was true in all experiments and a similar effect occurred in large fibres from spindles without the cold response.

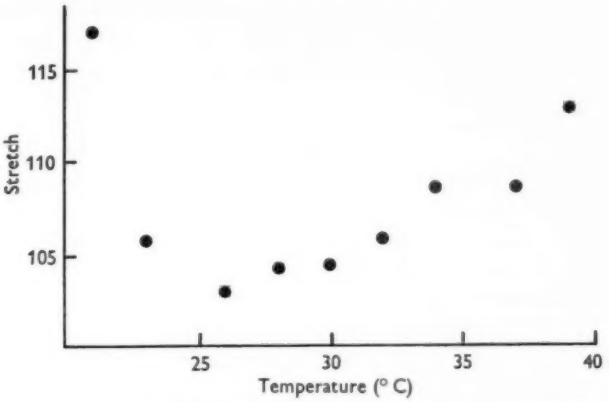


Fig. 11. Measurement of threshold to stretch sufficient for a maintained discharge of more than 10 sec at different temperatures. Stretch expressed as % over resting length. Fibre without cold response (see text).

The effect of calcium

Calcium is known to increase the stability of the membrane in nerve and muscle. Increasing the Ca^{2+} concentration in the Krebs's solution from 2·46 to 3·69 mM completely stopped the response to cold while the stretch response could still be elicited. The sensitivity of the receptors varied inversely with the Ca^{2+} concentration, but no precise measurements of threshold were made.

Reducing the Ca^{2+} concentration to 1·23 mM initially increased the sensitivity of the spindle to a given stretch (increasing the frequency and duration of the burst of impulses). Then, after approximately 30 min, the temperature and frequency range of the cold response was increased (Fig. 12).

Diminished Ca^{2+}, like increased K^+, made spindles innervated by large fibres, which had no cold response in normal Krebs's solution, develop one.

Receptor potentials

Receptor potentials were recorded by the method described by Lippold *et al.* (1960) at the beginning and end of the cold response. Figure 13

shows a record of the cold response at 32° C which was recorded close to the nerve ending. The receptor potential can be seen building up before each action potential. The discharge was just about to stop as a result of rewarming but in the interval where one spike has dropped out no abortive spike can be seen. The receptor potential is still present but is not large enough to generate a spike and there are no large oscillatory potentials.

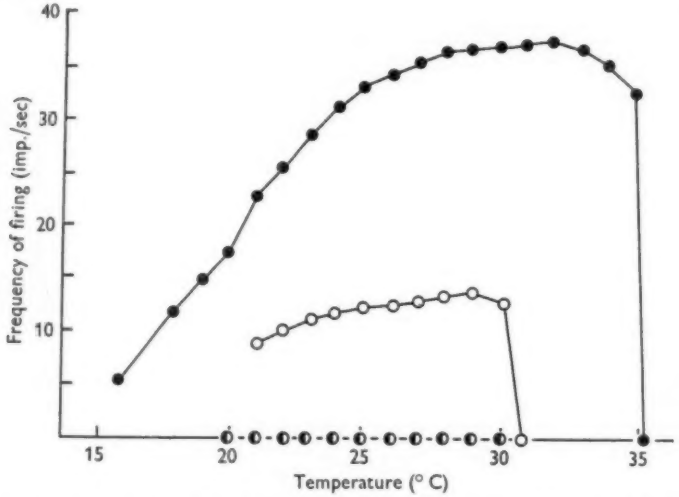


Fig. 12. The effect of calcium concentration on the frequency of firing at different temperatures; ○ in normal Krebs's solution containing 2·46 mM-$CaCl_2$; ◐ in 3·69 mM concentration; ● in 1·23 mM concentration.

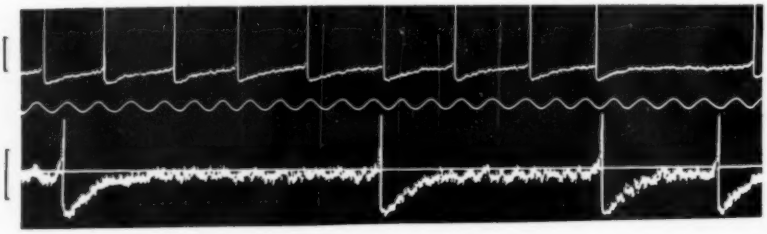


Fig. 13. Action potentials recorded close to nerve entry from a single spindle at 32° C when the cold response is about to stop on rewarming. Both records show action potentials dropped out. The receptor potentials are recorded but show no oscillations or abortive spikes during these gaps. Lower record gain increased 3× and sweep speed 2×. Voltage calibration, upper trace 200 μV, lower trace 100 μV. Time marker, upper trace, 50 c/s.

DISCUSSION

It is clear from our results that muscle spindles in the mammal fire spontaneously, in the absence of stretch, when they are brought to temperatures below 32° C. It might be expected that spindles would give rise to a brief discharge on rapid cooling, for Bernhard & Granit (1945) have described certain nerve fibres which respond to large, quick temperature changes by initiating several spikes. Muscle spindles, however, fire with a regular, non-adapting discharge, of frequency dependent upon the temperature and not its rate of change. The cold response of muscle spindles does therefore seem to be a specific effect of low temperature.

There are two possible explanations of this cold response in muscle spindles. One is that at low temperatures the physical properties of the muscle or the immediate surroundings of the nerve terminal are altered in such a way that a constant deformation of the sensitive membrane is produced. We have experimental evidence that this hypothesis is unlikely. All attempts to shorten further the relaxed muscle spindle, either by direct manipulation or by extrafusal twitches, fail to stop the cold discharge. In addition, the discharge is regular, of comparable frequency in different experiments and has a characteristic temperature–frequency curve which is similar to the discharge of a specific cold receptor (Hensel & Zotterman, 1951*a*). Cold cannot produce its effect in a specific cold receptor by a mechanical deformation of the sensory endings because these are not sensitive to mechanical stimulation (Dodt & Zotterman, 1952).

The second hypothesis which could be invoked to explain the cold response is that low temperatures alter the properties of the nerve terminal membrane. In the first place it is possible that the normal relation between membrane potential and firing rate, found by Katz (1950) to be linear, is altered or has a different slope in the cold. If this factor was solely responsible, it would be necessary to suppose that firing in the cooled terminal now occurred at the resting potential level. In other words there must be a reduction to zero in the threshold for firing, as a result of cooling. This would be similar to the spontaneous discharge observed when nerves are immersed in Ca^{2+}-free media. One might therefore expect that the sensitivity to a given depolarization or stretch would be greater on cooling.

In fact the converse is true. In our experiments a degree of stretch of the muscle which would evoke a high-frequency burst of action potentials at body temperature would only give a just detectable increase in firing rate at 20° C (when the cold discharge was present). However, in Ca^{2+}-free media at body temperature the spindles are firing spontaneously and their

sensitivity to stretch is increased. It is therefore unlikely that changes in the threshold for firing and in the sensitivity of the terminals can explain the production of the cold discharge.

A more attractive explanation is that low temperatures act by depolarizing the membrane of the nerve endings. All those factors which are known to depolarize membranes increase the discharge yet do not alter the general form of the relation with temperature. Furthermore, the same factors produce the characteristic discharge in spindles which otherwise do not have one. Conversely, hyperpolarizing the membrane reduces or abolishes the discharge. With present techniques it is not possible to demonstrate directly a depolarization due to cold; the nerve terminals are too small to enable intracellular recordings to be made.

It is interesting to observe that it is only in the case of recordings made from smaller fibres that this cold response can be obtained. This may reflect some functional or anatomical difference in the endings connected to the small fibre. Hensel & Zotterman (1951*b*) also found that pressure receptors with axons below about 10 μ in diameter would give a brief discharge when they were rapidly cooled, whereas those with axons of diameter greater than this did not do so. Douglas, Ritchie & Straub (1959) found that cooling caused a discharge in mammalian C fibres (as distinct from their endings) which depended upon their diameter. In the muscle spindle, however, it is the nerve ending which responds to cold, since separating the nerve from its receptor abolishes the discharge.

Since the cold discharge occurs in the anaesthetized but otherwise intact animal, it is relevant to consider its physiological implications. We do not at present know whether a continuous low-grade discharge of about 10–15/sec from more than half of the stretch receptors in a cooled limb would have any reflex or central effects.

SUMMARY

1. Spindles in the completely relaxed tenuissimus muscle of the cat gave a regular afferent discharge at temperatures below about 32° C, while still having a normal response to stretch.

2. Two-thirds of afferent fibres from stretch receptors had this property; those without the response invariably had the largest axons. In high K^+ or in low Ca^{2+} concentrations the large fibres developed a response to cold.

3. Factors which caused depolarization of the nerve ending (increased K^+ concentration, stretch, d.c. polarization) accelerated the discharge. Hyperpolarization (d.c. polarization, or low K^+ concentration) reduced or abolished the activity. On the other hand, full relaxation of the muscle or twitches of the extrafusal fibres did not affect the discharge.

4. The threshold to stretch also fell in the cold. This would be expected if depolarization of the membrane were occurring or its excitability were increased.

5. This response to cold is similar to that found in specific cold receptors.

We are indebted to the Nuffield Foundation for a grant in support of this work.

REFERENCES

ADRIAN, R. H. (1956) The effect of internal and external potassium concentration on membrane potential of frog muscle. *J. Physiol.* **133**, 631–658.

BERNHARD, C. G. & GRANIT, R. (1945). Nerve as a model temperature end organ. *J. gen. Physiol.* **29**, 257–266.

BUCHTAL, F. & JAHN, U. (1957). Spontaneous activity in isolated muscle spindles. *Acta physiol. scand.* **42**, Suppl. 145.

DODT, E. & ZOTTERMAN, Y. (1952). The discharge of specific cold receptors at high temperatures (the paradoxical cold). *Acta physiol. scand.* **26**, 358–365.

DOUGLAS, W. W., RITCHIE, J. M. & STRAUB, R. W. (1959). Discharges in non-myelinated (C) fibres in the cat's saphenous nerve in response to changing the temperature of the skin. *J. Physiol.* **146**, 47*P*.

HENSEL, H. & ZOTTERMAN, Y. (1951*a*). Quantitative Beziehungen zwischen der Entladung einzelner Kältefasern und der Temperatur. *Acta physiol. scand.* **23**, 291–319.

HENSEL, H. & ZOTTERMAN, Y. (1951*b*). The response of mechano-receptors to thermal stimulation. *J. Physiol.* **115**, 16–24.

HUNT, C. C. & KUFFLER, S. W. (1951*a*). Stretch receptor discharges during muscle contraction. *J. Physiol.* **113**, 298–315.

HUNT, C. C. & KUFFLER, S. W. (1951*b*). Further study of efferent small nerve fibres to mammalian muscle spindles. Multiple spindle innervation and activity during contraction. *J. Physiol.* **113**, 283–297.

KATSUKI, Y., YOSHIMO, S. & CHEN, J. (1950). Action currents of the single lateral-line nerve fibre of fish. I. On the spontaneous discharge. *Jap. J. Physiol.* **1**, 87–99.

KATZ, B. (1950). Depolarization of sensory terminals and the initiation of impulses in the muscle spindle. *J. Physiol.* **111**, 261–282.

LIPPOLD, O. C. J., REDFEARN, J. W. T. & VUČO, J. (1958). The effect of sinusoidal stretching upon the activity of stretch receptors in voluntary muscle and their reflex responses. *J. Physiol.* **144**, 373–386.

LIPPOLD, O. C. J., NICHOLLS, J. G. & REDFEARN, J. W. T. (1960). Electrical and mechanical factors in the adaptation of a mammalian muscle spindle. *J. Physiol.* **153**, 209–217.

MATTHEWS, B. H. C. (1933). Nerve endings in mammalian muscle. *J. Physiol.* **78**, 1–53.

J. Physiol. (1960), **153**, *pp.* 232–238
With 1 *text-figure*
Printed in Great Britain

VASOMOTOR FIBRES TO SKIN IN THE UPPER ARM, CALF AND THIGH

BY D. A. BLAIR, W. E. GLOVER AND I. C. RODDIE
From the Department of Physiology, The Queen's University of Belfast

(*Received* 30 *November* 1959)

Though the responses of the hand and forearm skin blood vessels to body heating and cooling are qualitatively similar there is a difference in the pattern of vasomotor control in the two areas. The blood vessels of the hand are normally subject to a high degree of vasoconstrictor tone even when the subject is comfortably warm. Blocking the nerves to the hand with local anaesthetic normally increases the blood flow through the hand to about 30–40 ml./100 ml./min (Roddie, Shepherd & Whelan, 1957*a*). The very large increase in hand blood flow during body heating seems due entirely to release of vasoconstrictor tone. Despite the evidence for vasodilator fibres to the hands of patients with Raynaud's disease (Lewis & Pickering, 1931), careful investigation has failed to provide any evidence that such fibres are involved in the vasodilatation during body heating in the hands of normal individuals. During body heating the blood flow in the normal hand does not exceed that in the nerve-blocked hand (Warren, Walter, Romano & Stead, 1942; Sarnoff & Simeone, 1947; Arnott & Macfie, 1948; Gaskell, 1956; Roddie, Shepherd & Whelan, 1957*c*) and atropinization of the hand tissues fails to reduce the vasodilatation (Gaskell, 1956; Roddie, Shepherd & Whelan, 1957*b*).

In forearm skin, however, the vessels are not subject to any appreciable vasoconstrictor or vasodilator tone when the subject is comfortably warm. When the cutaneous nerves to the forearm are blocked under these conditions there is little change in flow (Grant & Holling, 1938; Doupe, Cullen & Macaulay, 1943; Edholm, Fox & Macpherson, 1957). If the nerves are blocked when the subject is cold an increase in skin blood flow is observed, indicating the presence of vasoconstrictor fibres (Roddie, Shepherd & Whelan, 1957*d*). The fall in forearm blood flow during body cooling can be prevented by intra-arterial administration of bretylium tosylate, an adrenergic blocking agent (Blair, Glover, Kidd & Roddie, 1959) showing that the vasoconstrictor fibres in forearm skin are adrenergic. The increase in skin blood flow during body heating seems to be due to the activity of cholinergic nerves producing vasodilatation. It does not occur if the

sympathetic nerves to forearm skin are blocked (Grant & Holling, 1938; Doupe *et al.* 1943; Edholm *et al.* 1957; Roddie *et al.* 1957*d*) and it is reduced and delayed by atropinization of the forearm (Roddie *et al.* 1957*b*; Fox & Hilton, 1958). There is now evidence that the skin vasodilatation in the forearm during heating may be due to products of sweat-gland activity rather than to the excitation of specific vasodilator fibres (Fox & Hilton, 1958).

In view of the contrast between the vasomotor innervation of hand and forearm it seemed desirable to study the innervation of skin areas elsewhere in the limbs and the present paper describes some experiments which were designed with this end in view.

METHODS

Nine experiments were performed on six healthy young adults lying recumbent in a comfortably warm room (temperature 21–23° C). Subjects were lightly clad, but covered with a blanket. In any one experiment blood flow was measured bilaterally in either forearm, upper arm, calf or thigh. Forearm blood flow was measured by means of the water-filled plethysmographs described by Greenfield (1954). A larger plethysmograph with a suitably sized sleeve was used to measure flow in the calf and upper arm, and in thinner subjects, the thighs. In all experiments the plethysmograph was maintained at 35° C. During measurements the circulation to the limb distal to the plethysmograph was arrested by a cuff inflated to 200 mm Hg. A collecting cuff was applied just proximal to the plethysmograph. After the subject had rested in the laboratory for about 1 hr, resting blood flows were measured on the two sides. The position of the plethysmograph on the left limb was marked with a skin pencil, and the plethysmograph was then removed from the limb. The positions of the cutaneous nerves supplying the skin area under study were determined by faradic stimulation. The nerves were traced proximally up the limb and blocked by infiltrating 1–2 ml. of 2 % lignocaine containing adrenaline (1:80,000) around each of them. The block was performed as far away as possible from the plethysmograph area. When about 80 % or more of the skin area under study had become anaesthetic, the limb was replaced in its original position in the plethysmograph and the blood flow was again measured bilaterally at intervals until the end of the experiment. The anaesthesia of the skin area was checked at intervals throughout the experiment and, where necessary, further injections of local anaesthetic were made.

The subjects were then cooled for about 40 min. The blanket was removed and the room temperature was allowed to drop to 16–18° C. The subjects' feet (forearm and upper-arm experiments) or hands (calf and thigh experiments) were immersed in water at 17° C. Iced water was sprayed in a current of air from a vacuum cleaner pump over their naked chests and abdomens. Shivering frequently occurred in the first few minutes of cooling but then ceased and the subject was reasonably comfortable during the period in which observations were made.

Indirect heating was then applied. The room temperature was returned to 21–23° C. The subjects were wrapped in blankets and their feet and calves (upper arm and forearm experiments) or hand and forearm (thigh and calf experiments) were placed in stirred water at 44° C. Heating was continued for 40–60 min. This degree of heating usually causes an increase in body temperature of about 1° C and is accompanied by profuse sweating.

RESULTS

Figure 1 illustrates the result of an experiment in which blood flow was simultaneously measured in both forearms. At the beginning of the experiment, when the subject was comfortably warm, the blood flow through each forearm was about 4 ml./100 ml./min. Blocking the cutaneous nerves to the left forearm caused little change in the level of blood flow in either arm. Cooling the body caused a 58% fall in the blood flow in the normal forearm, but only a 12% fall on the nerve-blocked side. This is in

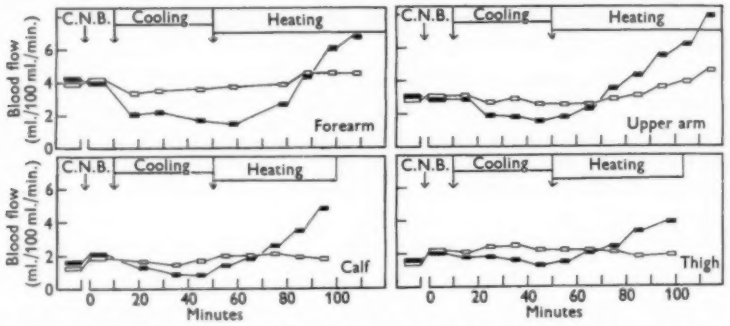


Fig. 1. The effects of body cooling and heating on blood flow in the normal and cutaneous-nerve-blocked forearm, upper arm, calf and thigh: ■ right side; □ left side. C.N.B. = cutaneous nerves on left side blocked with local anaesthetic.

keeping with the conclusion that the blood vessels in forearm skin are innervated with vasoconstrictor fibres which are activated in response to cooling the body (Roddie *et al.* 1957*c*, *d*). Heating the body caused a 93% increase in blood flow above the resting level in the normally innervated forearm, whereas it only increased by 12% on the nerve-blocked side. Visible sweating was observed in the normal though not the nerve-blocked forearm. This result confirmed the findings of Edholm *et al.* (1957), which indicated that the vasodilatation in forearm skin during body heating is due to an active vasodilator mechanism mediated through fibres running with the cutaneous nerves.

In four experiments of similar design the vasomotor innervation of the skin of the upper arm was studied (Fig. 1; Table 1). The pattern of result was similar to that found in the forearm. When the subject was comfortably warm, blocking the cutaneous nerves to the upper arm caused little change in blood flow, the average change being a fall of 0·2 ml./100 ml./min. However, when the subject was subsequently cooled, the average fall in blood flow in the nerve-blocked upper arm was only 12%, whereas in the normal upper arm the average fall was 67% of the resting level. Nerve block also depressed the vasodilatation during body heating. The

average increase in flow on the nerve-blocked side was 41 % above the resting level, whereas the average increase on the normal side was 79 %. It is likely that the difference would have been greater had the cutaneous nerve block been complete in all instances.

A similar pattern emerged in two experiments on the calf (Fig. 1; Table 1). Though cutaneous nerve block caused a small increase in blood flow (average 0·7 ml./100 ml./min), an increase (average 0·4 ml./100 ml./min) was also seen in the opposite (control) limb. Nerve block did not,

TABLE 1. The effects of body cooling and heating on blood flow in normal (control) limbs and limbs with the cutaneous nerves blocked (test limbs). The figures in the table are average values obtained for blood flow (ml./100 ml./min) during 5 min periods before nerve block, after nerve block, during body cooling and during body heating

		Control limb				Test limb			
Subject	Part studied	Before block of test limb	After block of test limb	During cooling	During heating	Before block of test limb	After block of test limb	During cooling	During heating
W.E.G.	Forearm	4·2	4·0	1·7	6·7	4·0	4·2	3·7	4·7
B.G.	Upper arm	4·2	4·0	0·8	7·0	4·0	3·7	2·7	4·8
L.A.	Upper arm	3·7	4·2	2·0	7·5	3·0	2·5	3·0	4·0
D.A.B.	Upper arm	3·0	2·9	1·4	8·5	3·0	3·0	2·5	4·9
I.C.R.	Upper arm	5·7	5·7	1·3	7·0	3·5	3·7	2·0	4·5
D.A.B.	Calf	3·6	3·6	1·8	6·5	3·6	4·3	3·7	3·0
W.E.G.	Calf	1·7	2·4	1·0	5·0	1·4	2·1	1·7	2·0
I.C.R.	Thigh	2·1	2·7	1·8	3·8	1·8	3·0	2·5	2·8
P.H.	Thigh	1·8	3·0	1·5	3·8	1·8	2·4	2·4	2·0

therefore, greatly alter the level of flow relative to that on the control side. Body cooling caused an average reduction of 53 % in the blood flow in the normal calf but only a 16 % reduction in the nerve-blocked calf. In response to body heating there was a 94 % increase in flow above the resting level on the normal side but there was no increase at all on the nerve-blocked side.

In two experiments on the thigh similar results were obtained (Fig. 1; Table 1). Though cutaneous nerve block resulted in a rise in blood flow, there was a similar increase on the normal side. Cooling the body resulted in an average fall in blood flow of 37 % on the normal side compared with a fall of only 7 % on the nerve-blocked side. Heating the body resulted in an average increase in flow of 36 % on the normal side, whereas the blood flow on the nerve-blocked side showed no increase.

It is clear, therefore, that in the upper arm, calf and thigh as in the forearm, cutaneous nerve block in a comfortably warm subject does not result in any important change in flow relative to that in the control limb. In response to body cooling and heating limb blood flow decreases and increases respectively. These changes in flow are depressed or abolished by blocking the cutaneous nerves to the part.

DISCUSSION

Cutaneous nerve block greatly reduced or abolished the changes in blood flow during body heating and cooling. It follows that the changes which normally occur are brought about by vasomotor nerves in skin. If methods are employed in the forearm which allow simultaneous estimation of skin and muscle blood flow, it can be shown that the increase in forearm blood flow during body heating is confined to the skin. For example, when skin and muscle blood flow in the forearm are simultaneously estimated by Hensel's heated thermocouples (Barcroft, Bock, Hensel & Kitchin, 1955), or by changes in the oxygen saturation of effluent blood from superficial and deep forearm veins (Roddie, Shepherd & Whelan, 1956), skin but not muscle flow increases during body heating. Body heating fails to cause an increase in forearm blood flow when the skin circulation is suppressed by adrenaline iontophoresis (Edholm, Fox & Macpherson, 1956). The present experiments indicate that a similar situation prevails in the upper arm, thigh and calf, and provides further evidence that the 'vasomotor centres' controlling skin vessels can function independently of those controlling muscle vessels.

The pattern of vasomotor control of skin in the upper arm, calf and thigh appears similar to that in the forearm. The finding that cutaneous nerve block caused little change in the blood flow in these areas indicated that the skin vessels were not subjected to any important vasoconstrictor or vasodilator tone when the subject was comfortably warm. Body cooling caused the blood flow on the normal side to fall below that on the nerve-blocked side, indicating the presence of vasoconstrictor fibres. During cooling the blood flow in the forearm, upper arm, calf and thigh fell to about half that of the previous resting level. This degree of reduction in flow has been obtained in the forearm by iontophoresis of adrenaline into the skin (Cooper, Edholm & Mottram, 1955) which has been shown to suppress, completely or almost completely, the cutaneous circulation (Edholm *et al.* 1956). It would appear from the present experiments that the activity of vasoconstrictor fibres in forearm skin can also almost completely suppress the skin circulation.

During body heating the blood flow on the normally innervated side rose substantially above that on the nerve-blocked side. This finding shows that the increase in blood flow that normally occurs is due to an active vasodilator mechanism. Though there is no evidence which definitely excludes the participation of specific vasodilator fibres in this response, it now seems more probable that the vasodilatation is due to release of bradykinin-forming enzyme from the activated sweat glands, and the subsequent formation of bradykinin (a polypeptide with vasodilator

properties) in the subcutaneous tissue space. Bradykinin-forming activity has been demonstrated in sweat collected from the human hand and forearm, and an increase in bradykinin activity occurs in the perfusate of the subcutaneous tissue space of the forearm during body heating (Fox & Hilton, 1958). There is a close analogy between the behaviour of the sweat glands and the salivary glands, where the vasodilatation associated with glandular activity has been shown to be due to release of bradykinin-forming enzyme from the activated gland (Hilton & Lewis, 1956). In certain animal species which lack eccrine sweat glands the skin vasodilatation in response to body heating seems to be due exclusively to release of vasoconstrictor tone (Green, Howard & Kenan, 1956). Finally, emotional sweating which occurs in the hand during stress is frequently accompanied by a vasodilatation (Allwood, Barcroft, Hayes & Hirsjärvi, 1959).

The present experiments indicate that the skin blood vessels of the upper arm, calf and thigh take part in temperature regulation. During body cooling the blood flow through these parts fell due to vasoconstrictor nerve activity, and during body heating it rose due an active vasodilator mechanism. The fact that the blood-flow changes with heating and cooling were of different sizes in different areas does not necessarily imply that there is any quantitative difference in the behaviour of the vasomotor nerves in these areas. It is possible that it is only a reflexion of the varying proportions of skin, muscle and bone in the different parts.

SUMMARY

1. Blood flow was measured bilaterally in the upper arm, calf or thigh of normal subjects by venous occlusion plethysmography.

2. When the subject was comfortably warm, blocking the cutaneous nerves to the experimental side with local anaesthetic caused little change in flow on the experimental side relative to that on the control side. It was concluded that the skin blood vessels in these areas, like those in the forearm, are not subjected to any important vasoconstriction or vasodilator tone under these conditions.

3. When the subject was subsequently cooled, the blood flow on the innervated side fell to a greater extent than on the nerve-blocked side. This result indicates the presence of vasoconstrictor fibres in the skin of the areas studied.

4. When the subjects were heated, the blood flow on the control side rose substantially above that on the nerve-blocked side, indicating that the vasodilatation was due to an active vasodilator mechanism mediated through fibres running with the cutaneous nerves.

REFERENCES

ALLWOOD, M. J., BARCROFT, H., HAYES, J. P. L. A. & HIRSJÄRVI, E. A. (1959). The effect of mental arithmetic on the blood flow through normal, sympathectomized and hyperhidrotic hands. *J. Physiol.* **148**, 108–116.

ARNOTT, W. M. & MACFIE, J. M. (1948). Effect of ulnar nerve block on blood flow in the reflexly vasodilated digit. *J. Physiol.* **107**, 233–238.

BARCROFT, H., BOCK, K. D., HENSEL, H. & KITCHIN, A. H. (1955). Die Muskeldurchblutung des Menschen bei indirecter Erwärmung und Abkühlung. *Pflüg. Arch. ges. Physiol.* **261**, 199–210.

BLAIR, D. A., GLOVER, W. E., KIDD, B. S. L. & RODDIE, I. C. (1959). The effect of bretylium tosylate (Darenthin) on certain vascular reflexes in human limbs. *J. Physiol.* **149**, 74–75*P*.

COOPER, K. E., EDHOLM, O. G. & MOTTRAM, R. F. (1955). The blood flow in skin and muscle of the human forearm. *J. Physiol.* **128**, 258–267.

DOUPE, J., CULLEN, C. H. & MACAULAY, L. J. (1943). Studies in denervation. F. The circulation in the skin in the proximal parts of the limbs. *J. Neurol.* **6**, 129–132.

EDHOLM, O. G., FOX, R. H. & MACPHERSON, R. K. (1956). The effect of body heating on the circulation in skin and muscle. *J. Physiol.* **134**, 612–619.

EDHOLM, O. G., FOX, R. H. & MACPHERSON, R. K. (1957). Vasomotor control of the cutaneous blood vessels in the human forearm. *J. Physiol.* **139**, 455–465.

FOX, R. H. & HILTON, S. M. (1958). Bradykinin formation in human skin as a factor in heat vasodilatation. *J. Physiol.* **142**, 219–232.

GASKELL, P. (1956). Are there sympathetic vasodilator nerves to the vessels of the hands? *J. Physiol.* **131**, 647–656.

GRANT, R T. & HOLLING, H. E. (1938). Further observations on the vascular responses of the human limb to body warming; evidence for sympathetic vasodilator nerves in the normal subject. *Clin. Sci.* **3**, 273–285.

GREEN, H. D., HOWARD, W. B. & KENAN, L. F. (1956). Autonomic control of blood flow in hind paw of the dog. *Amer. J. Physiol.* **187**, 469–472.

GREENFIELD, A. D. M. (1954). A simple water-filled plethysmograph for the hand or forearm with temperature control. *J. Physiol.* **123**, 62–64*P*.

HILTON, S. M. & LEWIS, G. P. (1956). The relationship between glandular activity, bradykinin formation and functional vasodilatation in the submandibular salivary gland. *J. Physiol.* **134**, 471–483.

LEWIS, T. & PICKERING, G. W. (1931). Vasodilatation in the limbs in response to warming the body; with evidence for sympathetic vasodilator nerves in man. *Heart*, **16**, 33–51.

RODDIE, I. C., SHEPHERD, J. T. & WHELAN, R. F. (1956). Evidence from venous oxygen saturation measurements that the increase in forearm blood flow during body heating is confined to the skin. *J. Physiol.* **134**, 444–450.

RODDIE, I. C., SHEPHERD, J. T. & WHELAN, R. F. (1957*a*). Humoral vasodilatation in the forearm during voluntary hyperventilation. *J. Physiol.* **137**, 80–85.

RODDIE, I. C., SHEPHERD, J. T. & WHELAN, R. F. (1957*b*). The contribution of constrictor and dilator nerves to the skin vasodilatation during body heating. *J. Physiol.* **136**, 489–497.

RODDIE, I. C., SHEPHERD, J. T. & WHELAN, R. F. (1957*c*). A comparison of the heat elimination from the normal and nerve-blocked finger during body heating. *J. Physiol.* **138**, 445–448.

RODDIE, I. C., SHEPHERD, J. T. & WHELAN, R. F. (1957*d*). The vasomotor nerve supply to the skin and muscle of the human forearm. *Clin. Sci.* **16**, 67–74.

SARNOFF, S. J. & SIMEONE, F. A. (1947). Vasodilator fibres in human skin. *J. clin. Invest.* **26**, 453–459.

WARREN, J. V., WALTER, C. W., ROMANO, J. & STEAD, E. A. (1942). Blood flow in the hand and forearm after paravertebral block of the sympathetic ganglia. Evidence against sympathetic vasodilator nerves in extremities of man. *J. clin. Invest.* **21**, 665–673.

J. Physiol. (1960), **153**, *pp.* 239–249
With 1 *text-figure*
Printed in Great Britain

TISSUE 5-HYDROXYTRYPTAMINE AND URINARY 5-HYDROXYINDOLEACETIC ACID AFTER PARTIAL OR TOTAL REMOVAL OF THE GASTRO-INTESTINAL TRACT IN THE RAT

By G. BERTACCINI
From the Institute of Pharmacology, University of Parma, Italy

(*Received* 11 *January* 1960)

It is generally accepted that the gastro-intestinal mucosa represents the most important site of production and storage of 5-hydroxytryptamine (5-HT) in mammals. In the rat, the total 5-HT in the gastro-intestinal tract may vary according to age, weight, strain, diet, and probably season, between 80 (Erspamer, 1954) and 300 μg/kg (Bertaccini, unpublished). Of this total amount, about 8–15 % is contained in the stomach, 30–40 % in the small intestine and the remaining 45–60 % in the large intestine.

On the basis of these data and the easy availability of a large number of uniform animals, rats seemed particularly suitable for studying the effect of removal of important portions of the 5-HT-secreting intestinal tissue or of the entire gastro-intestinal tract, on the serum and tissue levels of 5-HT as well as on the urinary excretion of 5-hydroxyindoleacetic acid (5-HIAA), the main metabolite of 5-HT.

METHODS

Experimental animals. The large intestines were removed from three groups of rats (300 in all) weighing 180–250 g. Each group consisted of animals of the same breed and sex, and of approximately the same age. The first experiment was carried out on male rats kept on a non-standard laboratory diet; the second on female rats, and the last on male Wistar rats. In the second and third experiments the rats were kept on the Rockland diet. Complete gastro-enterectomy was performed on 60 rats of both sexes; 30 rats were kept as controls.

Operation procedure and post-operative treatment

Removal of the large intestine. The rats were starved 24 hr before operation, but were allowed water *ad lib.*

The well known resistance of rat's peritoneal cavity against infections allowed the operation to be performed with care for cleanliness but without rigorous asepsis. The operative area was shaved and painted with 5 % tincture of iodine U.S.P.; penicillin 10,000 u./rat was given subcutaneously for 1 day before and 2 days after the operation, to reduce the risk of peritonitis due to leakage at the site of anastomosis.

Under light ether anaesthesia the abdomen was opened by a mid-line incision and the appendix was delivered from the peritoneal cavity on to saline packs. Beginning from the ileocaecal valve, all the blood vessels supplying the portion of the intestinal tract that was to

be excised were ligated. The large intestine was separated from its mesentery by careful dissection up to a point as near as possible to the anus, and the gut without its mesentery was thus gently excised with the minimum trauma and stretching. After the resection the continuity of the bowel was restored by an end-to-end anastomosis, with fine silk and a small curved needle. Side-by-side anastomosis was found to be more difficult to perform and less well tolerated. The abdomen was then closed with four or five interrupted sutures. Catgut was used for muscle layers and silk thread for skin. Some of the controls were subjected to simple laparotomy.

After the operation the animals were housed, in groups of two or three, in dry, clean cages. Twice in the first 24 hr 6 ml. of saline solution with 5% glucose was administered subcutaneously to each rat. From the second day on animals were offered milk and sugar, soaked bread and fresh liver in addition to amino-acid and vitamin mixtures. The rats were returned to their usual diet a fortnight after the operation. Intact and laparotomized control rats were always given the same diet as the operated ones.

The post-operative course was very satisfactory and the survival rate was 70–75%. The only trouble was a diarrhoea which lasted for about 1 month. Thereafter the stools became more formed. All animals lost weight during the first month, but then resumed normal growth rates comparable to, and in some instances greater than, in the controls. Operated rats showed, after recovery, a striking voracity.

As in the experiment of Clatworthy, Saleeby & Lovingood (1952), who examined dogs deprived operatively of 40–80% of the small intestine, Bertaccini, Nobili & Zamboni (1959) found that 3 months after the operation there was a significant rise in haemoglobin concentration and erythrocyte and platelet counts. In addition, operated rats presented a 35% increase in oxygen consumption above that of control rats and a slight rise (+0·6° C) in body temperature.

The animals were killed by decapitation at varying intervals after the resection. The viscera appeared quite normal and, except in rats operated 10 and 15 days before, no traces of intestinal suture were found. However, the stomach plus small intestine of operated rats showed a weight increase of 10–20% compared with the controls.

Total gastro-enterectomy. The animals were not starved before operation. Under ether anaesthesia a long, mid-line incision was made and wide exposure was obtained by the use of a retractor. First, gastrectomy was performed as described by Meli (1957). After this the bile duct was delivered and gently separated from mesentery and duodenal vessels. A small portion of mucosa of the duodenal loop including the outlet of the bile duct was isolated between clamps, severed and then inserted into the abdominal wall, to drain the bile externally. A single ligature was placed round the coeliac and the superior mesenteric arteries as near the aorta as possible. The rectum with its vessels was clamped, ligated distal to the clamp, and then cut between the clamp and the point of ligation. Finally, the whole gut, freed from its ligamentous and vascular attachments, was removed.

In some groups of rats, to avoid the contamination of urine from bile, which could interfere with the estimation of 5-HIAA, the bile duct was left free in the abdomen. After removing the gastro-intestinal tract, the abdomen was filled with sterile gauze. Penicillin 10,000 u./rat was given immediately after the operation, and 6 ml. of physiological saline solution with 5% glucose was injected subcutaneously every 6 hr. Because of the severity of this operation only 40–50% of the animals could be used for experiment. They were killed by decapitation 1–3 days later.

Analytic procedure

Preparation of acetone extracts. The following tissues and organs were examined: blood, gastro-intestinal tract, spleen, brain, lungs, liver, kidneys, ears and hind paws.

Blood was kept for 2–3 hr at room temperature, then during the night at +4° C. The serum was separated by centrifugation and then treated with 4 vol. of acetone. After standing during the night the liquid was filtered and the clear filtrate used for bioassay.

The gastro-intestinal tract was opened longitudinally, washed under running tap water and carefully dried with filter paper. It was minced with scissors and extracted with 4 parts (4 ml./g) of acetone for 24 hr, and then, after decanting the liquid, re-extracted for another 24 hr with 3–4 parts of 80% acetone. The combined filtrates were stored in the cold until used. All other tissues were treated in the same way but without washing. Before the bioassay acetone was removed by evaporation under reduced pressure at 40–50° C, or under an air stream in a boiling water-bath. The remaining aqueous liquid was brought to the desired volume with physiological saline solution.

Bioassay of 5-HT. Ovariectomized adult rats were injected once or twice with 50–100 μg oestradiol dipropionate 4–7 days before they were used. Uteri from these animals were suspended in a 10 ml. bath of atropinized (10^{-7}) Tyrode solution, kept at 30° C and bubbled through with air. The period of contact between uterus and the active solution (5-HT or tissue extract) was for 3–4 min and the interval between doses was 2–3 min. In the assays particular attention was paid not only to the heights of the contractions but also to how well they were maintained (Erspamer, 1940). The standard was 5-HT creatinine sulphate (Farmitalia S.p.A., Milan); the results, however, were always expressed in terms of 5-HT base.

Urine collection and estimation of urinary 5-HIAA. Groups of four rats were put into diuresis cages. Urine was collected in graduated cylinders containing 0·3 ml. of chloroform and 0·3 ml. of acetic acid, in order better to preserve the excreted 5-HIAA. At the end of the collection period the urine was either immediately frozen or treated with 1 vol. acetone which, of course, had to be removed by evaporation before the estimation of 5-HIAA. The acetone-urine was stored at −15° C. In preliminary experiments it was found that the content of 5-HIAA remained unchanged for at least 1 week in frozen urine, and for up to 15 months in urine plus acetone kept at −15° C. The 5-HIAA in urine was estimated by the colorimetric method of Macfarlane, Dalgliesh, Dutton, Lennox, Nyhus & Smith (1956).

Chromatographic detection of tryptophan and its metabolites. This was carried out in control as well as in operated rats after a loading dose of L-tryptophan (100 mg/kg by gastric tube). After the tryptophan administration the animals were housed in metabolism cages and stools and urine were collected separately for 24 hr. Faeces were weighed and extracted with 4 parts (4 ml./g) of acetone; urine, reasonably free from faecal contamination, with 1 part of acetone.

The concentrated extracts were chromatographed ascendingly on Whatman No. 1 paper. For unidimensional chromatograms the solvent was the *n*-butanol–acetic-acid–water mixture (4:1:5); for bidimensional chromatograms the same mixture was used for the first run and distilled water for the second. Tryptophan and other indoles were visualized by spraying with a 2% alcoholic solution of *p*-dimethylaminobenzaldehyde followed by exposure in a large glass chamber to HCl vapour; kynurenine and xanthurenic acid were visualized by their fluorescence reaction (Coppini, Benassi & Montorsi, 1958).

RESULTS

Tissue levels of 5-HT after removal of the large intestine. The results on 24 groups of rats are summarized in Table 1. The tissue contents of 5-HT were followed for up to 7 months after removal of the large intestines. In two other experiments carried out in different seasons, with male rats of different strain, results were obtained in substantial accordance with those shown in Table 1. Particular emphasis should be laid on the fact that brain 5-HT levels regularly presented a 20–30% decrease following removal of the large intestine.

TABLE 1. 5-HT content (μg/g or μg/ml.) of some tissues of the rat after removal of the large intestine. Each experiment was done on the pooled tissues from 4 rats. When an average is given the number of experiments is shown in brackets

	Days after the operation										
Tissue	Control 0*	10	15	30	Control 60†	60	90	Control 120†	120	Control 225†	225
Brain	0·26		0·23	0·25							
	0·27	0·17	0·24	0·32	0·50	0·22	0·25		0·32	0·36	0·20
	0·32	0·16	0·20	0·18	0·41	0·28	0·36	0·33	0·24	0·31	0·21
	0·30		0·21	0·25	0·36					0·29	
	0·30										
Stomach and small intestine	2·51 (6)	2·27 (2)	2·34 (4)	1·87 (4)	2·30 (3)	1·73 (2)	2·28 (2)	3·70	2·70 (2)	3·08 (2)	2·05
Serum	0·59 (6)	0·63 (2)	0·51 (4)	0·62 (4)	0·53 (3)	0·54 (2)	0·43 (2)	0·60	0·66 (2)	0·82 (3)	0·85 (2)
Spleen	2·25 (5)	2·60 (2)	1·67 (4)	2·05 (4)	2·96 (3)	2·70 (2)	2·79 (2)	1·93	1·79 (2)	2·43 (3)	2·00 (2)
Lungs	2·14 (6)	2·00 (2)	1·93 (3)	1·56 (4)	1·93 (3)	2·16 (2)	3·71 (2)	2·88	2·95 (2)	3·35 (3)	3·00 (2)
Liver	0·35 (3)	0·30 (2)	0·24 (2)	0·15 (2)	0·27 (3)	0·32 (2)	0·36 (2)	0·35	0·28 (2)	0·45 (3)	0·37 (2)
Kidneys	0·10 (2)	0·11 (2)	0·12 (2)	0·05	0·09 (2)	0·05	0·19 (2)	0·15	0·18 (2)	0·15 (2)	0·18
Ears	0·32 (2)	0·43	0·31 (2)	0·63 (2)	0·67 (3)	0·79 (2)	0·68 (2)	0·43	1·06 (2)	0·33 (3)	0·94 (2)
Paws	0·15 (5)	0·22 (2)	0·17 (4)	0·15 (4)	0·19 (3)	0·39 (2)	0·40 (2)	0·44	0·44 (2)	0·19 (3)	0·24 (2)

* Control rats killed at the beginning of the experiment. † Control rats killed 60, 120 and 225 days, respectively, from the beginning of the experiment.

The following conclusions may be drawn from the tabulated data:

(1) The 5-HT content of stomach and small intestine did not show significant changes at any time after operation. The observed fluctuations in the 5-HT levels were always within the normal range. In this respect it should be noted that the slight diminution in the 5-HT content, per gram of fresh tissue, of the small intestine of operated rats is compensated for by the 10–20% increase in the weight of the small intestine observed in these animals compared with the controls.

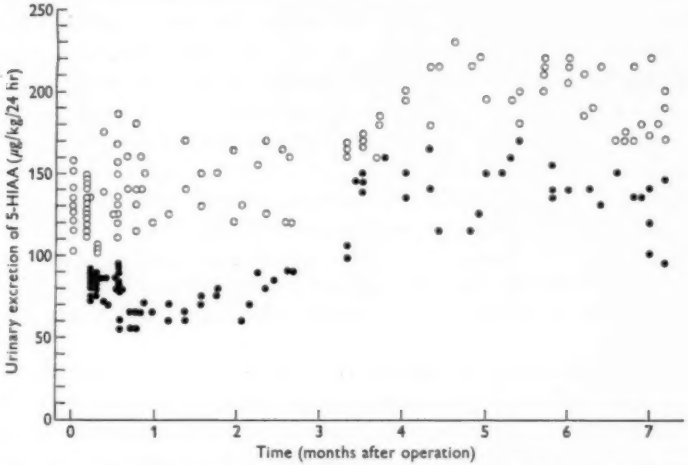


Fig. 1. Daily urinary excretion of 5-HIAA after removal of the large intestine; ○, controls, ● operated rats. Each value refers to the pool of urine obtained from four rats.

(2) The same may be said for serum and all other examined tissues except the brain. In some cases (e.g. lungs, liver, kidneys) a tendency to a lowering of 5-HT levels was observed, especially 15 and 30 days after the operation, but, owing to the considerable variations in the normal 5-HT content of rat tissues, it was impossible to ascribe any definite significance to the above occasional changes.

(3) The 5-HT content of the brain was significantly lower ($P < 0.01$) in operated animals than in controls.

5-HIAA excretion after removal of the large intestine. The results are shown in Fig. 1. The daily urinary excretion of 5-HIAA was determined at the beginning of the experiments both in control rats as well as in the rats which were later to be submitted to removal of the large intestine. Each value refers to the pool of urine obtained from four rats. It is clear from Fig. 1, which illustrates an experiment lasting more than 7 months,

that removal of the large intestine regularly produced a conspicuous decrease in the daily urinary excretion of 5-HIAA. This decrease was already evident in the first days after the operation and reached its maximum during the first 2 months, the values of urinary 5-HIAA being in the operated animals only 50 % of those found in the controls. It may also be seen from Fig. 1 that, whereas the values of 5-HIAA in control rats were rather scattered, those in operated rats were much less so. Later on, starting from the third month, the urinary excretion of 5-HIAA began to rise both in control and in operated animals. A maximum increase was reached in the fifth month, then the values tended to remain constant until the end of the experiment. It may be noted, however, that the percentage increase in the operated animals was more marked than that in the controls. In fact, whereas operated rats were excreting 65 μg/kg/24 hr on the 15th–60th days after the operation and 140 μg/kg/24 hr 4 months after the operation (more than 100 % increase) control rats were excreting 145 μg/kg/24 hr and 200 μg/kg/24 hr respectively (38 % increase). It is uncertain whether the general increase in 5-HIAA excretion, observed in the control rats during the course of our experiments, is due to the ageing of the animals (old rats contain in their tissues more 5-HT than young animals; Bertaccini, 1958*b*) or whether it is to be ascribed to seasonal variation.

Absorption of dietary tryptophan (TP) in normal and operated animals. L-tryptophan is the remote precursor of 5-HT, and hence of 5-HIAA. A deficiency in dietary TP produces in human beings and animals both a reduction in tissue 5-HT and in urinary 5-HIAA; an excess of dietary TP has the opposite effect (Stacey & Sullivan, 1957; Eber & Lembeck, 1958; Zbinden, Pletscher & Studer, 1958; Towsend, Katis & Sourkes, 1958).

It is well known that TP absorption takes place exclusively in the small intestine. Nevertheless, it seemed necessary to exclude the possibility that the reduced urinary 5-HIAA observed in our operated rats was at least partly due to a diminished intestinal absorption of TP. A number of rats were therefore given a dose of L-TP 100 mg/kg by stomach tube. It was possible to demonstrate by paper chromatography that absorption and metabolism of the amino acid were the same both in control and in operated animals. In fact, only traces of the administered TP were found in the stools and very small amounts in the urines. There were also no appreciable differences in the urinary excretion in xanthurenic and kynurenic acids between operated and control animals, as assessed by visual comparison, in Wood's light, of bidimensional chromatograms of urine. Since significant differences between control and operated rats could not be found in 5-HT content of the small intestines, the serum and other tissues (except brain), it may be concluded that the reduction in urinary

excretion of 5-HIAA after removal of the large intestine was not in any way due to deficiency in TP absorption or metabolism.

Tissue levels of 5-HT after removal of the entire gastro-intestinal tract. The results obtained are summarized in Table 2: in each experiment tissue from two to four rats was pooled. The main conclusions which may be drawn from the tabulated data are as follows: (*a*) In spite of acute removal of all the 5-HT-secreting cells of the gastro-intestinal mucosa, there was no appreciable change in 5-HT content in the brain, and in the ears and paws. (*b*) Serum, spleen and lungs showed a marked decrease in 5-HT content.

TABLE 2. 5-HT content (μg/g or μg/ml.) of some tissues of the rat after removal of the entire gastro-intestinal tract. Each experiment was done on the pooled tissues from 2–4 rats. When an average is given the number of experiments is shown in brackets

		Operated animals, days after operation		
Tissue	Controls	1	2	3
Brain	0·33 (5)	0·24	0·33 (5)	0·26
Serum	0·82 (3)	0·43	0·27 (2)	0·14
Spleen	2·40 (5)	2·00	1·43 (5)	1·30
Lungs	3·00 (5)	1·60	1·50 (5)	0·76
Ears	0·35 (5)	1·20	0·57 (4)	—
Paws	0·25 (5)	0·22	0·29 (4)	0·13

5-HIAA excretion after removal of the entire gastro-intestinal tract. The results shown in Table 3 demonstrate that removal of the entire gastro-intestinal tract produced a tremendous decrease in the urinary excretion of 5-HIAA. In the first 24 hr period the urine contained barely 10% of the normal 5-HIAA, in the second 24 hr period less than 3–4%. Urine volume was of course greatly reduced, but the urinary excretion of 5-HIAA in a 24 hr period has been shown to be largely independent of the quantity of urine: in fact hydrated rats and rats deprived of water excreted approximately the same amount of 5-HIAA.

Urinary excretion of 5-HIAA following tryptophan administration in gastro-enterectomized rats. As was previously stated, gastro-enterectomy produced a striking reduction in the urinary excretion of 5-HIAA. It then seemed worth while to establish whether this was due only to removal of the gastro-intestinal 5-HT-secreting tissue or also to lack of L-TP in other tissues which may be capable of a complete synthesis of 5-HT, e.g. brain tissue and mast cells. Twelve operated rats were therefore given 4 subcutaneous doses each of L-TP 100 mg/kg, the first immediately after operation, and then 4, 8 and 12 hr later. Eight intact rats were subjected to the same treatment. Intact rats, and operated rats which were not given tryptophan, acted as controls.

It was observed that the administration of L-TP, while causing a slight

increase (up to 20%) in the intact rats, did not produce any increase in the urinary 5-HIAA of operated rats, which excreted < 10% of the normal 5-HIAA. Hence, it does not seem probable that lack of L-TP had played any important part in the reduction of urinary 5-HIAA observed in gastro-enterectomized animals.

TABLE 3. Daily urinary excretion of 5-HIAA (μg/kg) after removal of the entire gastro-intestinal tract. Each value refers to the pooled urine obtained from 2–4 rats

Before operation		After operation	
		0–24 hr	25–48 hr
200	210	14	6
190	170	12	4
185	220	11	4
205	195	26	4
190	—	12	—
158	—	27	—
210	—	18	—
150	—	15	—
182	—	12	—
170	—	16	—
Means 184	198	16·3	—

DISCUSSION

The present experiments have demonstrated that removal of the large intestine in rats, while leaving virtually unaffected the 5-HT content of nearly all the tissues examined, produced a conspicuous decrease in the 5-HIAA of the urine. Because no alterations in the absorption and metabolism of dietary L-TP and consequently no deficiency of L-TP, could be demonstrated, it is highly probable that the reduction in urinary 5-HIAA excretion was due to a diminished 5-HTP (5-hydroxytryptophan) and 5-HT biosynthesis, caused by a reduction of the 5-HT-secreting tissue in the gastro-intestinal tract, namely the enterochromaffin cells. The percentage decrease in urinary 5-HIAA excretion, especially in the first 2 months after the operation, agrees satisfactorily with the reduction in the 5-HT-secreting tissue provoked by removal of the large intestine. The obvious conclusion is that biosynthesis of 5-HT is carried out mainly in the mucosal enterochromaffin cells, and that urinary 5-HIAA is derived mainly from 5-HT of intestinal origin, as is well known (Erspamer, 1940; Toh, 1954; Dalgliesh & Dutton, 1957*a*, *b*; Haverback & Davidson, 1958; Bertaccini, 1958*a*; Rosenberg, Davis, Moran & Zimmermann, 1959; Erspamer & Testini, 1959; Bertaccini & Chieppa, 1960). Total gastro-enterectomy produced a virtual disappearance of urinary 5-HIAA, in spite of repeated parenteral administration of very high doses of L-TP. This represents strong additional evidence in favour of the hypothesis that 5-HT is the specific hormone of the entero-chromaffin cell system

(Erspamer & Asero, 1952), and proves also that, under normal conditions, practically all urinary 5-HIAA is in fact derived from the 5-HT of intestinal origin.

Removal of the large intestine, which, as already stated, might cause even a 50 % reduction in the urinary excretion of 5-HIAA, did not produce any significant change in the 5-HT content either of blood serum (i.e. platelets) or of other tissues, except brain. Thus, in spite of reduced 5-HT secretion by the enterochromaffin cells, platelets while passing through the small intestine absorb from the plasma enough 5-HT to maintain their normal content of 5-HT. It is well known that they can absorb 5-HT against a gradient of 170/1 (Stacey, 1956). In fact, the 5-HT level of the part of the gastro-intestinal tract which was left *in situ* after operation was practically normal. This is in sharp contrast with the decreased 5-HT content of the gastro-intestinal mucosa in rats on a low-tryptophan diet (Eber & Lembeck, 1958).

Since liver, lungs, ears and paws of operated animals showed a practically normal level of 5-HT, it is evident that in these tissues too a normal absorption of 5-HT from blood or, more likely, a normal biosynthesis of 5-HT, has taken place, also in spite of a reduced biosynthesis of the amine by the enterochromaffin tissue. It should be noted that in the above rat tissues 5-HT is mainly localized in mast cells, which synthesize 5-HT from L-TP (Schindler, Day & Fischer, 1959).

Total gastro-enterectomy produced a significant decrease in the 5-HT content of blood serum, spleen and lungs, but not a total disappearance of the substance, as in the case of urinary 5-HIAA.

Removal of the large intestine provoked in the rat a significant lowering of the 5-HT content of brain tissue. The reason for this remains largely obscure. It may be that the decrease of brain 5-HT is somehow correlated with the increase in basal metabolism and body temperature observed by Bertaccini *et al.* (1959) in operated animals. Gastro-enterectomized rats, even when killed 2–3 days after operation in extremely bad condition, showed a virtually normal brain 5-HT content. The most likely interpretation of this fact is that brain tissue carries out a normal biosynthesis of 5-HT independently of the gut. Other possibilities are not easy to accept considering that the half-life of brain 5-HT is believed to be approximately 10–30 min (Udenfriend & Weissbach, 1958), so that in the 48-hr survival period the cerebral 5-HT ought to have been renewed 50–150 times.

Although the results obtained in the present investigation are, on the whole, in favour of the hypothesis that brain 5-HT is completely of local origin, other alternatives should be kept in mind. In fact, besides the intestine, the rat contains another important source of 5-HT in the mast

cells. Under these circumstances it is evidently impossible to decide whether the 5-HT found in the brain after total gastro-enterectomy is produced in the brain itself from L-TP, or whether it is derived by decarboxylation of 5-HTP offered to the cerebral tissue by the mast cells.

SUMMARY

1. Removal of the large intestine and total gastro-enterectomy have been carried out in rats with the purpose of investigating the effects of reduction or absence of the 5-hydroxytryptamine-secreting enterochromaffin cells on the biosynthesis of 5-hydroxytryptamine (5-HT).

2. Removal of the large intestine produced a considerable decrease in the daily 5-hydroxyindoleacetic acid (5-HIAA) urinary excretion, independent of disturbances in tryptophan absorption of metabolism. The 5-HT content of blood and other tissues examined did not show any appreciable change. The only exception was the brain, the 5-HT level of which was reduced.

3. Total gastro-enterectomy caused a virtual disappearance of 5-HIAA from the urine and a considerable lowering of the 5-HT in blood, spleen and lungs. No change in the 5-HT level was observed in the brain, paws and ears.

4. The enterochromaffin cells of the gastro-intestinal mucosa therefore represent the main source of 5-HT in the rat and practically the only source of blood 5-HT and of urinary 5-HIAA. The results obtained in gastro-enterectomized rats minimize the role of mast cells in the general metabolism of 5-HT in rats, since, in spite of repeated parenteral administration of L-tryptophan, these rats excreted $< 10\%$ of the normal 5-HIAA.

5. The biosynthesis of 5-HT in the brain seems largely independent of that in the gastro-intestinal mucosa. The significance of the decrease in brain 5-HT after removal of the large intestine is obscure. The maintenance of a normal 5-HT content in the brain during the 24–72 hr period following total gastro-enterectomy supports the hypothesis that neural 5-HT is of local origin.

This work was supported by a grant from the Rockefeller Foundation, New York.

REFERENCES

BERTACCINI, G. (1958*a*). Effect of removal of large segments of rat intestine on the biosynthesis and metabolism of 5-hydroxytryptamine. *Naturwissenschaften*, **45**, 548.

BERTACCINI, G. (1958*b*). Osservazioni sul contenuto in 5-idrossitriptamina di vari tessuti del ratto durante lo sviluppo post-natale. *Ricerca scient.* **11**, 2261–2263.

BERTACCINI, G. & CHIEPPA, S. (1960). Urinary excretion of 5-hydroxindoleacetic acid after removal of the large intestine in man. *Lancet*, **278**, 881.

BERTACCINI, G., NOBILI, M. B. & ZAMBONI, P. (1959). Influenza dell'asportazione dell' intestino crasso sul consumo di O_2 del ratto. *Arch. it. Sc. Farmacol.* **9**, 394–398.

CLATWORTHY, H. W., SALEEBY, R. & LOVINGOOD, C. (1952). Extensive small bowel resection in young dogs: effect on growth and development; experimental study. *Surgery*, **32**, 341–351.

COPPINI, D., BENASSI, C. A. & MONTORSI, M. (1958). Determinazione quantitativa di metaboliti del triptofano in liquidi biologici. *Atti Soc. Natur. Matem.* **89**, 50–53.

DALGLIESH, C. E. & DUTTON, R. W. (1957*a*). Biogenesis of 5-hydroxytryptophan. *Brit. J. Cancer*, **11**, 296–309.

DALGLIESH, C. E. & DUTTON, R. W. (1957*b*). On the site of formation of 5-hydroxytryptophan. *Biochem. J.* **65**, 22–23*P*.

EBER, O. & LEMBECK, F. (1958). Hydroxytryptamingehalt des Rattendarmes bei verschiedenem Tryptophangehalt der Nahrung. *Pflüg. Arch. ges. Physiol.* **265**, 563–566.

ERSPAMER, V. (1940). Pharmakologische Studien über Enteramin. I Über die Wirkung von Acetonextrakten der Kaninchenmagenschleimhaut auf den Blutdruck und auf isolierte überlebende Organe. *Arch. exp. Path. Pharmak.* **196**, 343–365.

ERSPAMER, V. & ASERO, B. (1952). Identification of enteramine, the specific hormone of the enterochromaffin cell system, as 5-hydroxytryptamine. *Nature, Lond.*, **169**, 800–801.

ERSPAMER, V. (1954). Il sistema cellulare enterocromaffine e l'enteramina (5-idrossitriptamina). *R.C. sci. Farmitalia*, **1**, 1–193.

ERSPAMER, V. & TESTINI, A. (1959). Observations on the release and turnover rate of 5-hydroxytryptamine in the gastrointestinal tract. *J. Pharm.* **11**, 618–623.

HAVERBACK, B. J. & DAVIDSON, J. D. (1958). Serotonin and the gastro-intestinal tract. *Gastroenterology*, **35**, 570–578.

MACFARLANE, P. S., DALGLIESH, C. E., DUTTON, R. W., LENNOX, B., NYHUS, L. M. & SMITH, A. N. (1956). Endocrine aspects of argentaffinoma, with special reference to the use of urinary 5-hydroxyindoleacetic acid estimation in diagnosis. *Scott. med. J*, **i**, 148–155.

MELI, A. (1957). Una tecnica semplificata per la gastrectomia totale nel ratto. *Arch. Sci. biol.* **41**, 394–396.

ROSENBERG, J. C., DAVIS, R., MORAN, W. H. & ZIMMERMANN, B. (1959). Serum serotonin concentration and urinary 5-hydroxyindoleacetic acid excretion following intestinal obstruction and resection. *Fed. Proc.* **18**, 503.

SCHINDLER, R., DAY, M. & FISCHER, G. A. (1959). Culture of neoplastic mast cells and their synthesis of 5-hydroxytryptamine and histamine in vitro. *Cancer Res.* **19**, 47–51.

STACEY, R. S. (1956). Red cells and 5-hydroxytryptamine. *J. Physiol* **132**, 39–40*P*.

STACEY, R. S. & SULLIVAN, T. J. (1957). Effect of diet and antibiotics on intestinal 5-hydroxytryptamine. *J. Physiol.* **137**, 63–64*P*.

TOH, C. C. (1954). Release of 5-hydroxytryptamine (serotonin) from the dog's gastrointestinal tract. *J. Physiol.* **126**, 248–254.

TOWSEND, E. E., KATIS, J. G. & SOURKES, T. L. (1958). Effect of tryptophan deficiency in rat on liver tryptophan peroxidase-oxidase (TPO) and urinary 5-hydroxy-3-indoleacetic acid (HIAA). *Fed. Proc.* **17**, 324.

UDENFRIEND, S. & WEISSBACH, H. (1958). Turnover of 5-hydroxytryptamine (serotonin) in tissues. *Proc. Soc. exper. Biol. Med, N.Y.*, **97**, 748–751.

ZBINDEN, G., PLETSCHER, A. & STUDER, A. (1958). Alimentäre Beeinflussung der enterochromaffinen Zellen und des 5-Hydroxytryptamingehaltes von Gehirn und Darm. *Z. ges. exp. Med.* **129**, 615–620.

J. Physiol. (1960), **153**, *pp.* 250–264
With 6 text-figures
Printed in Great Britain

ON THE EXCITANT EFFECT OF ACETYLCHOLINE ON STRUCTURES IN THE PREGANGLIONIC TRUNK OF THE CERVICAL SYMPATHETIC: WITH A NOTE ON THE ANATOMICAL COMPLEXITIES OF THE REGION

By W. W. DOUGLAS, D. W. LYWOOD and R. W. STRAUB
From the Department of Pharmacology, Albert Einstein College of Medicine, Yeshiva University, New York 61, *U.S.A.*

(*Received* 25 *January* 1960)

Numerous experiments have shown that acetylcholine (ACh) can initiate afferent impulses by an action on the terminals of a variety of sensory nerves including mechanoreceptors, thermoreceptors and chemoreceptors. It has been suggested that this action reflects the normal participation of ACh in the initiation of sensory impulses; but experiments with ACh antagonists render this improbable (see Gray, 1959) and a more common view is that this action of ACh is a pharmacological phenomenon reflecting some specialization of the receptor membrane for sensory function.

It is, however, possible that these effects of ACh have a more general significance and indicate a property of all nerve endings, efferent as well as afferent. While it is not clearly established that ACh excites efferent endings directly, some evidence to this effect has been obtained in several studies on different preparations. Coon & Rothman (1940), for example, have concluded that the piloerection which follows intradermal injection of ACh is attributable to the initiation of an axon reflex in sympathetic post-ganglionic fibres; and Masland & Wigton (1940) have suggested, on the basis of action potential studies on ventral spinal roots, that ACh injected close-arterially into a muscle may act directly on motor nerve terminals to evoke antidromic impulses. Neither type of experiment can, however, be taken as convincing evidence of a direct, excitant action of ACh on efferent nerve terminals. It has been argued that the piloerector effect does not involve sympathetic nerves, but some other unknown nervous elements (Brenning, 1956); and there is evidence that drugs may set up antidromic impulses in motor neurones indirectly through their effects on the skeletal muscle (Eccles, Katz & Kuffler, 1942; Lloyd, 1942).

In more recent experiments on cats Riker & Szreniawski (1959) have provided what appeared to be new evidence for a direct stimulant effect of ACh on efferent nerve terminals. They found that ACh injected intra-

arterially into the common carotid artery regularly induced action potentials in the preganglionic trunk below the superior cervical ganglion, even when the trunk had been severed from its central connexions, and their analysis led them to conclude that the effect was due to ACh acting on presynaptic endings. Since these results have a bearing not only on the problem of the special organization of the membrane at nerve terminals, but also, as stressed by Riker & Szreniawski, on the mechanisms of cholinergic transmission, we have carried out the present experiments in order to examine further the excitant action of ACh on preganglionic structures. Our experiments demonstrate a similar effect of ACh in rabbits, but have led us to a different interpretation of this phenomenon.

METHODS

All experiments were performed on adult rabbits of the New Zealand white variety anaesthetized with urethane (1·6–1·8 g/kg) given intravenously in a 25% (w/v) solution. A skin incision was made from the sternum along the length of the neck toward the tip of the mandible and a tracheal cannula was tied in. The right common carotid artery was exposed along its whole length in the neck by tying and cutting the muscles lying over it. The area was then flooded with liquid paraffin and dissection was continued under a binocular microscope giving $\times 6$ or more magnification.

Tying of large arteries. The purpose of this dissection was to minimize as far as possible the shunting effect of the large arteries which, when present, produced the result that only a small fraction of any substance injected into the carotid artery reached the superior cervical ganglion. In each experiment all the major branches of the external carotid artery were ligated as far cranially from the superior cervical ganglion as possible and in many experiments the internal carotid artery was also tied off at a level about 5 mm or more above the cranial pole of the superior cervical ganglion. Care was taken during ligation of large blood vessels to avoid any disturbance of the blood vessels supplying the superior cervical ganglion. One artery to the ganglion could usually be seen coming off the ascending pharyngeal artery and others seemed to reach the ganglion from the internal carotid artery well proximal to the point at which it was tied. This blood supply agrees with the scheme reported by Chungcharoen, Daly & Schweitzer (1952). These arteries to the superior cervical ganglion were not the only branches left patent. Considerable numbers of small vessels which supplied other tissues in the region of the superior cervical ganglion were left untied and, indeed, the bulk of the blood flow through the common carotid artery at the end of the dissection appeared to pass through these. It was deemed inadvisable to attempt to tie them off, for this would have risked injury to the superior cervical ganglion.

Preparation of nerves. During the course of the dissection and ligature of the large arteries the opportunity was taken to identify branches of the superior cervical ganglion which were appropriate for electrical recording. The most suitable post-ganglionic branch was then dissected free, carefully cleaned and occasionally desheathed for electrical recording. The branch chosen was usually one which lay superficially on the external carotid artery, but sometimes a branch which ran from the superior cervical ganglion medially towards the prevertebral muscles was used. The cervical sympathetic trunk was then cut low in the neck, usually about 1 cm above the thoracic inlet and some 4–5 cm below the superior cervical ganglion, i.e. well above the middle cervical and stellate ganglia. The vagus and aortic nerves were also tied and cut at the same level. The subsequent dissection of the cervical sympathetic trunk below the superior cervical ganglion varied from experiment to experiment, depending

upon whether it was intended to leave its circulation intact or not, and upon whether it was intended to record from a long or short length of nerve.

In some experiments the major part of the trunk below the superior cervical ganglion and above the level of section low in the neck was left undisturbed in its connective-tissue investment so that there was a continuing circulation of blood through it. In such experiments only that part of the cervical sympathetic trunk which lay immediately caudal to the superior cervical ganglion was freed from the surrounding tissues, and then only for a length sufficient to accommodate one or two pairs of recording electrodes, i.e. about 1–1·5 cm. When the sympathetic trunk was prepared in this way, arterial blood gained access to it not only from vessels in the connective-tissue sheath caudal to the recording electrodes, but also from vessels descending in the nerve sheath from the superior cervical ganglion and arising usually from the same small branch of the pharyngeal artery which entered the superior cervical ganglion at its lower pole.

In other experiments the cervical sympathetic trunk was cut high in the neck, leaving only a length of about 10–15 mm, attached to the superior cervical ganglion, to which the recording electrodes could be applied. Usually, when this was done, steps were taken to limit access of blood to the stump of cervical sympathetic trunk being recorded from. To this end the small blood vessels that could be identified entering the trunk at its upper end were tied off. Usually there were from two to four of them which could readily be identified and tied under × 16–25 magnification.

Injections. Acetylcholine was injected as the chloride in the buffered Locke's solution described below. It was injected into the carotid artery through a fine cannula made from a 25 or 28 gauge needle attached to a length of polythene tubing and inserted into the central end of the tied lingual branch of the external carotid artery. Injections were thus given retrogradely without occluding the common carotid artery, into the more or less complete cul-de-sac resulting from the ligature of the major arteries. They reached the sympathetic trunk and the superior cervical ganglion through the small arteries we have described. In those experiments in which the internal carotid artery was tied usually injection of 0·2–0·3 ml. sufficed to ensure exposure of the ganglion to the drug. When the internal carotid artery was not tied, however, 0·5 or even 1 ml. was sometimes required.

Perfusion. In some experiments, after the preparation had been made in the way just described the common carotid artery was tied low in the neck and the prepared superior cervical ganglion region was perfused with phosphate-buffered Locke's solution through a polythene cannula tied into the common carotid artery immediately cranial to the occluding ligature. The Locke's solution was oxygenated, had a pH of close to 7·2, and was delivered under a pressure of 80 mm Hg. It had the following composition (mM): NaCl 154; KCl, 5·6; $CaCl_2$, 2·2; Na_2HPO_4, 2·15; NaH_2PO_4, 0·85; glucose, 10. When perfusion was established, the animal was killed. The flow through the perfused region was quite small, usually about 1 ml./min, and was allowed to escape into the dead animal's venous bed. The inferior vena cava was cut to avoid any build up of venous pressure. In these experiments injections were made as in the normally circulated preparation, i.e. through a cannula inserted into the central stump of the lingual artery. This retrograde technique allowed an immediate displacement of the perfusion fluid and a prompt access of the injected material to the superior cervical ganglion. Since the injected volume was 1 ml. and greatly exceeded the combined dead spaces of the injection cannula and the perfused carotid region, the superior cervical ganglion was exposed to the injected solution for about 1 min. At any time after an injection was made, it could be removed by withdrawing 2 ml. or more of fluid through the injection cannula, and this was usually done unless a prolonged action was desired. In all the perfusion experiments the room temperature was kept at 36–37° C, so that this was the temperature of the preparation, the perfusion fluid and the injections.

Recording and stimulating. Action potentials were recorded through bright platinum electrodes and capacity-coupled amplifiers. The frequency response of these amplifiers was

limited by low- and high-frequency shunts, such as to halve the amplitude of signals at 35 c/s and at 500 or 2000 c/s. Electrical shocks were provided by a stimulator giving rectangular pulses of current and were applied to the platinum electrodes through a radio-frequency isolation unit (Schmitt, 1948). The electrical activity in pre- and post-ganglionic branches was displayed simultaneously on two oscilloscope beams and photographed on one moving film.

RESULTS

Discharges set up by acetylcholine in the preganglionic trunk

The discharges that ACh set up in the cervical sympathetic trunk in our experiments could be grouped into those which appeared to be ascending in the trunk, i.e. towards the superior cervical ganglion, and those which appeared to be descending in it, i.e. towards the thorax.

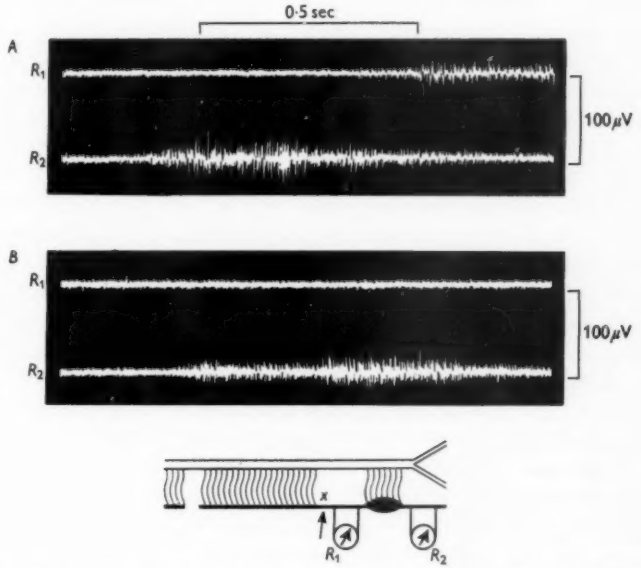


Fig. 1. Discharges set up by ACh in pre- and post-ganglionic branches of cervical sympathetic nerve with intact blood supply, and the effect of dividing the sympathetic trunk below the recording electrodes. Recording pairs of electrodes are on the preganglionic trunk immediately caudal to the superior cervical ganglion (R_1) and on a post-ganglionic branch (R_2). The interruption in the preganglionic trunk shows where it was cut low in the neck. Cross-hatching between the cervical sympathetic and the carotid artery indicates intact connective tissue sheath and blood vessels. *A*, effect of intracarotid injection of ACh 150 μg; action potentials appear in the post-ganglionic branch (R_2) and also, with delay, in the preganglionic trunk (R_1). Between *A* and *B* a second transection of the sympathetic trunk at *x*, just below R_1. *B*, effect of ACh 3 mg: the discharge appears in the post-ganglionic (R_2) but not in the preganglionic trunk (R_1).

Ascending discharges. In all the experiments the cervical sympathetic trunk was transected low in the neck. With recording electrodes placed on the cervical sympathetic trunk caudal to the superior cervical ganglion, action potentials were usually observed on injecting ACh into the carotid artery, provided the sympathetic trunk below the recording electrodes was left undisturbed down to its point of transection. The discharge was generally of much smaller amplitude than that recorded at the same time from a post-ganglionic branch, and was frequently delayed in its onset (Fig. 1*A*). The dose of ACh required to elicit it varied from animal to animal and to a greater extent than did the dose required to evoke a post-ganglionic discharge. Sometimes it occurred with the same dose as required to evoke a post-ganglionic discharge, but often it occurred only with doses several times greater.

In these experiments it seemed likely that the injected ACh had reached the sympathetic trunk below the recording electrodes as well as the region of the superior cervical ganglion, because most of the blood vessels to the trunk had been spared by the dissection. Two types of experiment showed that the activity observed did indeed arise caudally to the recording electrodes.

In the first type of experiment, after ACh had been found to initiate a discharge in the cervical sympathetic below the superior cervical ganglion, the trunk was cut caudal to the recording electrodes (at x in Fig. 1) and ACh was again injected. It was found that the preganglionic effect could then no longer be evoked, although a discharge could still be observed in the post-ganglionic branch. This pattern persisted even when much higher doses of ACh were given (Fig. 1*B*).

In the second type of experiment a somewhat longer length of the cervical sympathetic trunk below the ganglion was prepared and two pairs of electrodes were placed on it (Fig. 2, diagram). When ACh had been injected and noted to set up activity which was recorded at both these electrodes (Fig. 2*B*), the nerve was cut between the two pairs of electrodes (at x) and ACh was again injected. A brisk discharge of action potentials now appeared (Fig. 2*D*) at the caudal pair of electrodes (R_2), but not at the upper electrodes (R_1) nearer the ganglion.

In these experiments the activity recorded in the sympathetic trunk upon injecting ACh was clearly arising in the trunk itself and not in the superior cervical ganglion. It could not, therefore, have been due to antidromic firing from presynaptic endings in the superior cervical ganglion. The most probable explanation of the effect is that it was due to ACh stimulating aberrant ganglion cells. A number of reports have described such cells either scattered along the length of the cervical sympathetic trunk below the superior cervical ganglion or grouped together as an

accessory ganglion, usually in the middle third of the trunk (see Discussion). In the present experiments we were able to confirm the occurrence of such aberrant ganglion cells by using the electrophysiological techniques described by Douglas & Ritchie (1956).

In one experiment ACh was observed to initiate a well-marked discharge in the cervical sympathetic trunk below the superior cervical ganglion, even after the trunk had been separated from the adjacent tissues up to the superior cervical ganglion, and after the larger blood vessels descending

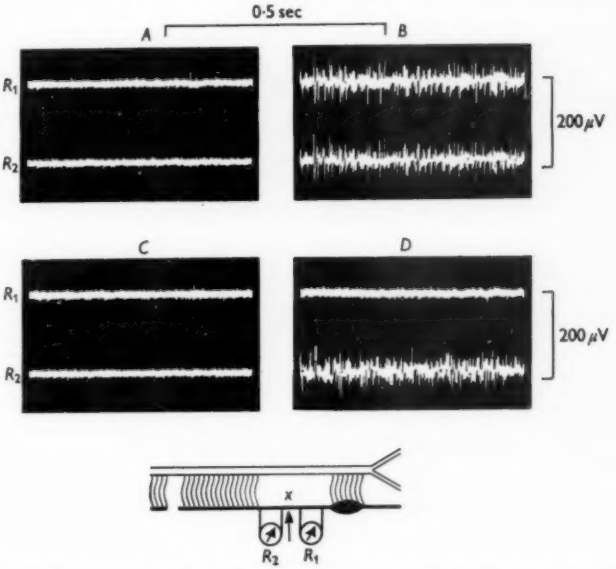


Fig. 2. Discharges set up by ACh in the cervical sympathetic trunk below the superior cervical ganglion, and effect of dividing the trunk between the two pairs of recording electrodes. In this and all subsequent figures the conventions used in the diagram are those adopted in Fig. 1. *A*, control records. *B*, 2 sec after injection of ACh 50 μg: similar discharges are recorded at both electrodes R_1 and R_2. Between *B* and *C* a second transection of the cervical sympathetic trunk at *x* between the two pairs of electrodes. *C*, controls. *D*, responses 2 sec after another injection of ACh (5 mg): a discharge appears at R_2, but not at R_1.

in its sheath had been ligated. In this, a perfused preparation, ACh 10 μg/ml. elicited a small response in the post-ganglionic trunk only. A higher concentration (250 μg/ml.) gave rise to a more pronounced discharge in the post-ganglionic trunk and a just detectable discharge in the preganglionic trunk. With an extremely high dose of ACh (25 mg/ml.) this preganglionic discharge became quite obvious, although still feeble in comparison with the post-ganglionic discharges seen previously with ACh

in a concentration one hundred times smaller. This effect, illustrated in Fig. 3, is one of the two largest (the other being that of Fig. 4) which we ever observed in preparations in which the cervical sympathetic trunk had been freed, except for its connexions with the superior cervical ganglion, and where its principal blood vessels had been tied off. In such preparations, as we will describe later, ACh usually failed to excite impulses in the preganglionic trunk. In this experiment the effect could again be accounted for by ganglion cells. These were shown to be present by the following experiment. Electrical shocks were applied near the cut end

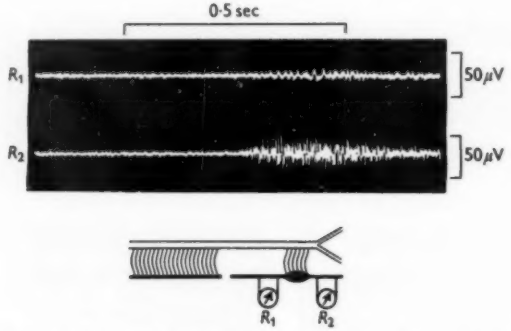


Fig. 3. Discharges set up by 25 mg ACh in a cervical sympathetic trunk which was cut and freed up to the superior cervical ganglion. The discharge in the preganglionic trunk (R_1), although one of the two largest observed in these experiments, is of small amplitude compared with the post-ganglionic discharge (R_2).

of the freed preganglionic trunk and the compound action potential was led off at the recording electrodes nearer the superior cervical ganglion. It was found that weak stimuli of 0·01 msec duration evoked not only a B but also a C potential. The C potential began to appear at the recording electrodes with intensities of stimulation sufficient to activate only the more excitable of the B fibres and it grew *pari passu* with the B potential as stronger shocks were applied. Douglas & Ritchie (1956) have analysed this phenomenon and have shown it to indicate the presence of synapses, preganglionic fibres of type B impinging upon post-ganglionic C fibres. There was no doubt that ACh could have gained access to these ganglion cells, for despite the fact that the principal blood vessels in the cervical sheath had been tied off, droplets of fluid gathered at the cut end of the nerve during the course of the experiment.

Descending discharges. In experiments in which the possibility of exciting the ganglion cells caudal to the recording electrodes was excluded by cutting away all the nerve below these electrodes, ACh usually failed to set up any activity in the stump of the trunk which was left connected with the

superior cervical ganglion (see p. 258). In a few experiments, however, it did cause a discharge. Since discharges in such preparations were seen only occasionally, it seemed unlikely that they were due to antidromic activity in preganglionic fibres, for then they would have been expected to occur in all these experiments in which the conditions were the same. A more probable explanation seemed to be that the activity was in 'aberrant' post-ganglionic fibres such as have been reported sometimes to run caudally in the cervical sympathetic trunk from cells in the superior

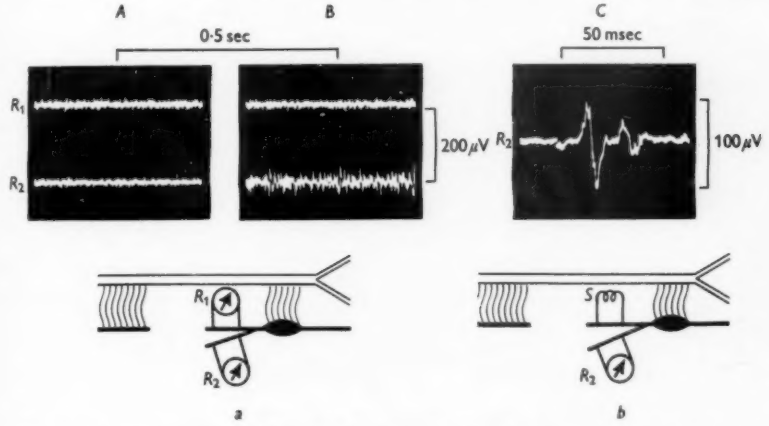


Fig. 4. Comparison of the effect of ACh on two fascicles of the preganglionic trunk in a perfused preparation. Recording electrodes R_1 and R_2 are placed on two fascicles of the sympathetic trunk freed up to the superior cervical ganglion. *A*, control. *B*, injection of 100 μg ACh: activity appears only in one fascicle (R_2). *C*, compound action potential recorded at R_2 on stimulating at *S* (the former site of R_1).

cervical ganglion (see Discussion). In one experiment, in which the effect was pronounced, and where the cervical sympathetic trunk split readily into fascicles, it proved possible to analyse it. In this experiment the 'preganglionic' cervical sympathetic trunk was split into two fascicles and a pair of recording electrodes was placed on each (Fig. 4*a*). When acetylcholine was injected, it set up activity in one only of these fascicles (Fig. 4*B*). The fascicle which was unresponsive to ACh was then stimulated electrically (see *b*). This evoked a compound action potential in the other fascicle, i.e. that which had responded previously to ACh (Fig. 4*C*). When, however, the stimulating and recording electrodes were interchanged, so that now stimuli were applied to the fascicle previously responsive to ACh, and records were taken from the other, a compound action potential could not be detected. Clearly, the fascicle which had responded to ACh contained descending post-ganglionic fibres that were activated by the ascending

preganglionic fibres in the other fascicle. In this experiment, therefore, the discharges set up in the cervical sympathetic trunk below the superior cervical ganglion could readily be accounted for by post-ganglionic fibres running caudally in the trunk from the superior cervical ganglion.

In one experiment we recorded from the cervical sympathetic trunk immediately above its point of section low in the neck. The sympathetic trunk above the recording electrodes was undisturbed and had its connective-tissue sheath intact up to the level of the superior cervical ganglion, but was transected just below that ganglion. When ACh was injected, a small discharge of impulses was recorded at the electrodes on the lower end of this cervical sympathetic trunk isolated from its principal ganglia. Clearly, these impulses could not be attributed to antidromic firing in preganglionic fibres ending in the superior cervical ganglion.

Failure of acetylcholine to excite antidromic impulses in preganglionic fibres

It was found that the response of preganglionic fibres to ACh could usually be tested after taking two precautions to avoid spurious results due to stimulation of aberrant ganglion cells. First, the recording electrodes were placed on the cervical sympathetic trunk no further than 5 mm below the superior cervical ganglion, and all the sympathetic trunk below the recording electrodes was cut away. Secondly, the access of injected ACh to the stump of 'preganglionic' trunk was minimized by tying off all the blood vessels entering it which could be seen under $\times 25$ magnification.

Experiments with the circulation intact. In seven experiments on superior cervical ganglia with their natural blood supply, clear-cut discharges were recorded in the post-ganglionic nerves on injection of 25–50 μg of ACh, provided all the major branches of the common carotid artery were tied. When the internal carotid artery was not tied, somewhat higher doses (up to 150 μg) were required. None of these injections elicited any discharge in the cervical sympathetic trunk below the ganglion. Nor was activity detected in the preganglionic trunk with doses of ACh 10, 20, or 100 times greater (Fig. 5*A–C*).

Experiments on perfused superior cervical ganglia. In two perfused preparations ACh 1–10 μg/ml. elicited a discharge in the post-ganglionic branch. In the first the effect could be obtained repeatedly with 10 μg/ml., but neither this concentration nor one a thousand times greater initiated any detectable discharge in the preganglionic trunk. In the second (Fig. 5*D*, *E*), post-ganglionic discharges were obtained with ACh 1 μg/ml. However, neither this dose nor doses of ACh ten or ten thousand times greater caused any discharge in the preganglionic trunk.

In the experiments on perfused ganglia we tested the effect of ACh after inhibiting cholinesterase with eserine sulphate (10^{-5} g/ml.) perfused through the ganglion for about 10 min before the ACh was injected. It was found that, although ACh could then initiate discharges in the post-ganglionic nerves in concentrations ten times smaller than those effective before eserine, there were again no discharges in the preganglionic trunk in either

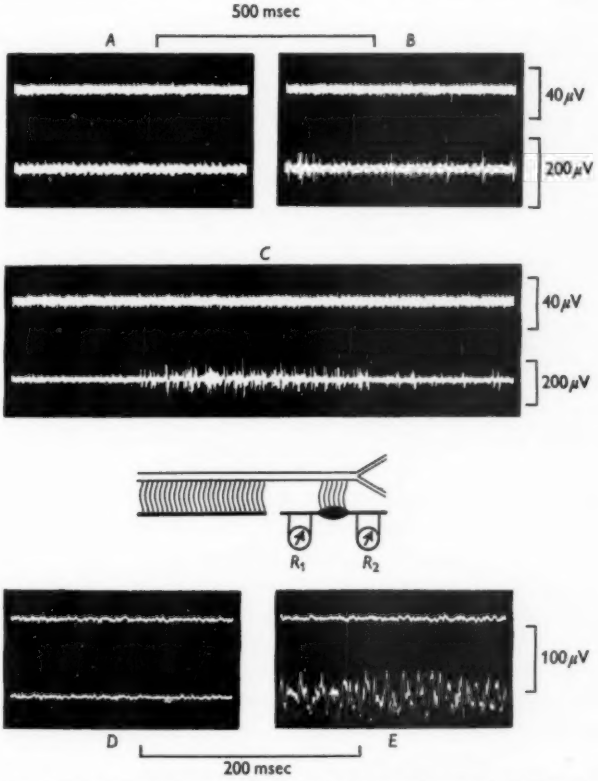


Fig. 5. Two preparations showing failure of ACh to initiate activity in the preganglionic fibres of the cervical sympathetic trunk. One recording electrode, R_1, is on the preganglionic trunk close to the superior cervical ganglion; the other, R_2, on a post-ganglionic branch. *A*, *B*, *C*, from a preparation with normal blood supply. *A*, control. *B*, 25 μg ACh; threshold response in the post-ganglionic branch only. *C*, a much higher dose of ACh (2·5 mg) initiates a discharge, again confined to the post-ganglionic branch. Note that the preganglionic recording was made at about 5× higher gain. *D* and *E*, from a perfused preparation. *E* shows effect of an enormous dose of ACh (10 mg/ml.) involving only the post-ganglionic branch (compare with the control *D*). In this preparation the threshold dose for post-ganglionic discharge was 1 μg/ml.

of the two experiments. Even when enormous doses of ACh were given there was still no obvious preganglionic effect in one preparation and only a very small and delayed discharge in the other.

DISCUSSION

Anatomy of the cervical sympathetic trunk. It is known that ganglion cells occur along the length of the cervical sympathetic trunk between the stellate and the superior cervical ganglia. Thousands of such aberrant ganglion cells may be present, both diffusely scattered or gathered into aggregations which sometimes form ganglia visible to the naked eye, as in the accessory cervical ganglion, which is of common occurrence (Langley, 1893; Douglas & Ritchie, 1956). The post-ganglionic fibres arising from these various cells occasionally leave the trunk as visible grey rami, but more often they run in the trunk cranially or caudally, intermingled with the preganglionic fibres supplying the superior cervical ganglion. In addition to these fibres, there may be others which descend caudally from ganglion cells lodged in the superior cervical ganglion. While all these arrangements, which have been schematized in Fig. 6, may not occur in every animal, they are common and some are to be expected in both cats and rabbits. Clearly, the 'preganglionic' cervical sympathetic nerve may contain ascending or descending post-ganglionic fibres.

Discharges initiated by acetylcholine in the cervical sympathetic trunk. The discharges evoked by ACh in the cervical sympathetic trunk in our experiments seem explicable on the above anatomical grounds. Most of the discharges were found to be ascending towards the superior cervical ganglion and appeared to be due to ACh exciting aberrant ganglion cells scattered in the trunk (*H*, Fig. 6) or in the accessory cervical ganglion (Fig. 6*C*, *E* and *F*). The discharges we occasionally observed when recording from a short length of the cervical sympathetic trunk immediately caudal to the superior cervical ganglion seemed attributable to the presence of caudally projected post-ganglionic fibres (Fig. 6*G*). Certainly this was demonstrated in one instance where a favourable anatomical arrangement permitted analysis (Fig. 4).

The question arises whether similar stimulation of aberrant ganglion cells accounts for the effect of ACh which was observed by Riker & Szreniawski (1959) in the cat's cervical sympathetic trunk and which they believed to be antidromic impulses in preganglionic fibres. It is probable that in Riker & Szreniawski's experiments the ACh injected into the carotid artery gained access to the cervical sympathetic trunk over most of its length, for they freed it from its investing tissue only over the lower 7–8 mm in the neck. Since histological evidence shows that this trunk in the cat also contains numerous caudally projected axons (Fig. 6*D*, *H*),

it would seem that caudally running impulses in post-ganglionic fibres are to be expected after ACh injection in such experiments.

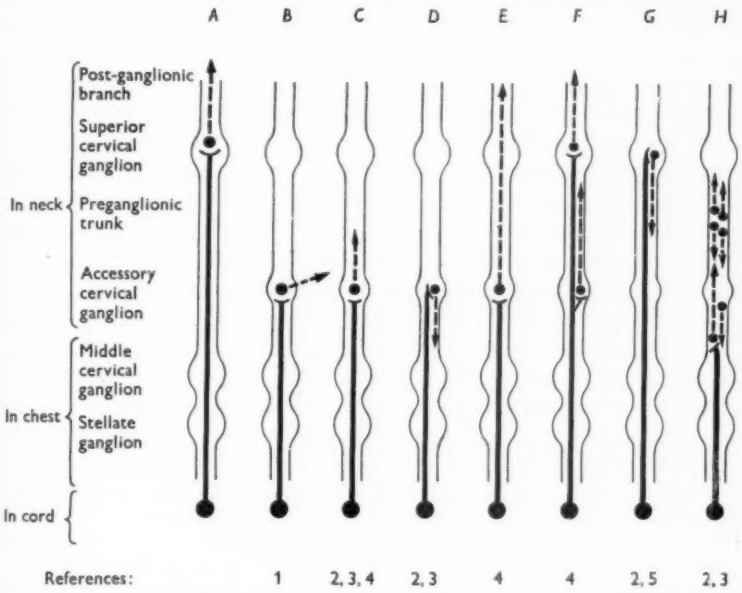


Fig. 6. Diagrams showing arrangements of pre- and post-ganglionic fibres in the cervical sympathetic trunk. *A*, the conventional arrangement. *B–H*, alternative arrangements known to occur. Preganglionic fibres are indicated by the solid lines; post-ganglionic fibres by the interrupted lines. The numbers below each diagram refer to the original papers and the animals studied: 1, Langley (1893), cat. 2, Foley (1945), cat. 3, Butson (1950), cat; rabbit. 4, Douglas & Ritchie (1956), rabbit. 5, this paper, rabbit.

Riker & Szreniawski do not discuss the evidence for such cells, but they do consider the possibility that their results might have been due to some hypothetical post-ganglionic fibres. These are represented (their Fig. 2*b*) as originating in ganglion cells that also send axons into the internal and external carotid nerves. Since they observed that discharges might occur in these post-ganglionic branches of the cervical sympathetic ganglion without a simultaneous discharge in the cervical sympathetic trunk, they rejected the possibility that cells with caudally projected axons are responsible for the discharges they observed in the preganglionic trunk. There is no evidence that the same ganglion cells which send axons caudally also send axons cranially; if we make the simpler assumption that they do not, it is easy to account for the quantitative differences which Riker & Szreniawski observed, for these may merely reflect the different numbers and situations of the ganglion cells and their accessibility to injections. Such quantitative differences may also explain the observation of Brown & MacIntosh (1938) that intracarotid injection of ACh in the cat 'sets up a discharge only in the post-ganglionic sympathetic trunk'. We usually required higher doses of ACh to excite the ganglion cells in the cervical sympathetic trunk, where the circulation is sluggish, than those in the superior cervical ganglion, where it is brisk.

The insensitivity of preganglionic endings to acetylcholine. While the presence of abundant ganglion cells in the complex arrangements shown in Fig. 6 makes it difficult to test how the preganglionic terminals in the superior cervical ganglion react to ACh, it appears that in the rabbit the test can be made by taking some simple precautions. When these were taken, it was clear that ACh did not elicit any detectable discharge in the cervical sympathetic trunk below the superior cervical ganglion, even when given in amounts from ten to ten thousand times greater than those eliciting discharges in its post-ganglionic branches. It seems that these preganglionic endings in the rabbit are highly resistant to the excitant action of ACh. The same appears true of the preganglionic fibres to the stellate ganglion of the cat. After ACh, Brink, Bronk & Larrabee (1946) observed discharges in its post-ganglionic branches with 25 μg/ml., but no discharges in its preganglionic branches even with 500 μg/ml. It appears to us that the only conclusion justified by their results and ours is that ACh fails to initiate impulses in preganglionic fibres even when given in amounts grossly in excess of those exciting ganglion cells.

In discussing the earlier experiments in which no preganglionic activity was apparent in response to ACh, Riker & Szreniawski have argued that conceivably the recording techniques used may have been inadequate, and unable to deal with 'the relative asynchrony in the acetylcholine-induced discharge' in the preganglionic trunk. This argument is unconvincing, for discharges were readily detected in the preganglionic nerves in the earlier experiments in response to potassium excess or calcium deprivation, and there is no reason to believe that such discharges were any more synchronous. It appears that the only reason Riker & Szreniawski have for assuming that the preganglionic discharge to ACh is 'relatively asynchronous' is that it was always of smaller amplitude than the discharge in the post-ganglionic branches. While this assumption allows them to reconcile the smallness of the preganglionic discharge with their generalization that the characteristic effect of ACh at the ganglionic synapse is to induce repetitive activity in the presynaptic as well as the post-synaptic nerves, there is no evidence for it. The smallness of the signals seems to accord better with the different explanation we have offered, i.e. that the discharge in the preganglionic trunk results from activity in only a fraction of the fibres and that those are post-ganglionic fibres of the thin non-myelinated type.

The finding that ACh over an enormous dose range is unable to initiate impulses from preganglionic endings would seem to set these endings apart from the other efferent cholinergic endings to skeletal muscles and sweat glands, which do appear to be excited by ACh. The difference may be one of degree only, for ACh does not seem regularly to excite antidromic impulses in these motor fibres. Although Masland & Wigton (1940) reported 'showers' of impulses in ventral root fibres when ACh was injected into the muscles, Diamond (1959) has recorded only feeble effects involving a few fibres; and while Coon & Rothman (1941) observed axon reflex sweating to ACh, this was less obvious in experiments by Collins, Sargent & Weiner (1959). The inexcitability of the preganglionic endings

to ACh might possibly be due to their exceptionally rich investment of cholinesterase; McIsaac & Koelle (1959) postulate such a protective role for this cholinesterase. Our experiments with eserine, however, suggest that cholinesterase is not a major factor in preventing antidromic impulses. A further possibility is that the behaviour of preganglionic endings reflects their anatomical arrangement in relation to the post-synaptic structures rather than the properties of the endings themselves. It has been argued that antidromic impulses occurring in motor nerves to skeletal muscle after the application of eserine are secondary to muscular activity; that it is, in fact, the electrical currents generated in the muscle which trigger off the impulses from the motor nerve endings (Eccles *et al.* 1942; Lloyd, 1942). The antidromic impulses seen after ACh might well have a similar explanation and be secondary to effector organ activity. In a study on regenerating nerve endings remote from the organs they normally innervate, Diamond (1959) found that, although sensory endings were readily excited by ACh, motor nerve endings were not. It seems an open question whether ACh can indeed excite any antidromic impulses in cholinergic nerves without the intervention of currents developed in post-synaptic structures. The anatomical relation between preganglionic endings and ganglion cells may not be favourable for such stimulation.

SUMMARY

1. In view of reports that acetylcholine sets up antidromic activity from a variety of efferent nerve endings, experiments have been performed on rabbits to test the sensitivity of preganglionic endings to acetylcholine.

2. When acetylcholine was injected into the common carotid artery, impulses appeared in the nerves above and below the superior cervical ganglion, i.e. both in its post- and preganglionic branches.

3. Electrophysiological analysis of the preganglionic trunk corroborated and extended previous evidence of its anatomical complexity and showed it to contain ganglion cells and post-ganglionic fibres.

4. The discharges set up by acetylcholine in the 'preganglionic' trunk all seemed explicable on the grounds that they were post-ganglionic discharges in aberrant fibres of this kind.

5. In experiments in which precautions were taken to avoid discharges in such aberrant post-ganglionic fibres, no activity could be detected in preganglionic fibres either in normally circulated or in perfused ganglia with doses of acetylcholine ten to ten thousand times greater than those exciting ganglion cells.

6. It is concluded that few, if any, sympathetic preganglionic fibres are excited to discharge antidromic impulses when their endings are exposed

to injected acetylcholine. This behaviour is compared with that of sensory endings and other efferent endings.

This investigation was supported by research grants (B 1093 and B 1319) from the National Institute of Neurological Diseases and Blindness of the National Institutes of Health, United States Public Health Service.

REFERENCES

BRENNING, R. (1956). The pilomotor axon reflex. *Acta Soc. Med. upsalien.* **61**, 227–251.

BRINK, F., BRONK, D. W. & LARRABEE, M. G. (1946). Chemical excitation of nerve. *Ann. N.Y. Acad. Sci.* **47**, 457–485.

BROWN, G. L. & MACINTOSH, F. C. (1938). Discharges in nerve fibres produced by potassium ions. *J. Physiol.* **96**, 10–11*P*.

BUTSON, A. R. C. (1950). Regeneration of the cervical sympathetic. *Brit. J. Surg.* **38**, 223–239.

CHUNGCHAROEN, D., DALY, M. DE B. & SCHWEITZER, A. (1952). The blood supply of the superior cervical sympathetic and the nodose ganglia in cats, dogs and rabbits. *J. Physiol.* **118**, 528–536.

COLLINS, K. J., SARGENT, F. & WEINER, J. S. (1959). Excitation and depression of eccrine sweat glands by acetylcholine, acetyl-β-methylcholine and adrenaline. *J. Physiol.* **148**, 592–614.

COON, J. M. & ROTHMAN, S. (1940). The nature of the pilomotor response to acetylcholine; some observations on the pharmacodynamics of the skin. *J. Pharmacol.* **68**, 301–311.

COON, J. M. & ROTHMAN, S. (1941). The sweat response to drugs with nicotine-like action *J. Pharmacol.* **73**, 1–11.

DIAMOND, J. (1959). The effects of injecting acetylcholine into normal and regenerating nerves. *J. Physiol.* **145**, 611–629.

DOUGLAS, W. W. & RITCHIE, J. M. (1956). The conduction of impulses through the superior cervical and accessory cervical ganglia of the rabbit. *J. Physiol.* **133**, 220–231.

ECCLES, J. C., KATZ, B. & KUFFLER, S. W. (1942). Effect of eserine on neuromuscular transmission. *J. Neurophysiol.* **5**, 211–230.

FOLEY, J. O. (1945). The components of the cervical sympathetic trunk with special reference to its accessory cells and ganglia. *J. comp. Neurol.* **82**, 77–92.

GRAY, J. A. B. (1959). 'Initiation of impulses at receptors' in *Handbook of Physiology*, Section 1: Neurophysiology, vol. I, p. 139, Ed. FIELD, J.: American Physiological Society, Washington, D.C.

LANGLEY, J. N. (1893). On an 'accessory' cervical ganglion in the cat and notes on the rami of the superior cervical ganglion. *J. Physiol.* **14**, i-ii.

LLOYD, D. P. C. (1942). Stimulation of peripheral nerve terminations by active muscle. *J. Neurophysiol.* **5**, 153–165.

MASLAND, R. L. & WIGTON, R. S. (1940). Nerve activity accompanying fasciculation produced by prostigmin. *J. Neurophysiol.* **3**, 269–275.

MCISAAC, R. J. & KOELLE, G. B. (1959). Comparison of the effects of inhibition of external, internal and total acetylcholinesterase upon ganglionic transmission. *J. Pharmacol.* **126**, 9–20.

RIKER, W. K. & SZRENIAWSKI, Z. (1959). The pharmacological reactivity of presynaptic nerve terminals in a sympathetic ganglion. *J. Pharmacol.* **126**, 233–238.

SCHMITT, O. H. (1948). A radio frequency coupled tissue stimulator. *Science*, **107**, 432.

J. Physiol. (1960), **153**, *pp.* 265–289
With 6 text-figures
Printed in Great Britain

THE TRANSMISSION OF HOMOLOGOUS SERUM PROTEINS TO THE FOETUS AND TO THE AMNIOTIC FLUID IN THE RHESUS MONKEY

By D. R. BANGHAM

From the National Institute for Medical Research, Mill Hill, London, N.W. 7

(*Received* 3 *February* 1960)

Electrophoretic studies of sera of foetuses show different absolute and relative concentrations of serum proteins compared with the maternal sera (Josephson & Gyllenswärd, 1957). Certain proteins have been described which occur in foetuses but not in adult animals (Pedersen, 1947; Scheinberg, Cock & Murphy, 1954; Deutsch, 1954; Dancis, Braverman & Lind, 1957; Bergstrand & Czar, 1957, 1958; Myant & Osorio, 1958), and certain constituents of adult serum do not occur in the foetus (Ewerbeck & Levens, 1950). The pattern of proteins changes with development of the foetus and the neonate. With the exception of antibodies, little is known of how much and which of the definitive serum proteins of the foetus are derived from the mother in primates. The transfer of antibodies from mother to young before and after birth has been studied in many laboratory animals and in man. Brambell (1958) has recently extensively reviewed this field. Nearly all the evidence for their transfer to the foetus in mammals (other than ungulates) is based on immunological studies comparing the titre of specific antibodies in maternal blood with their titre in foetal or cord blood.

The transmission of other serum proteins has received little attention. Heterologous albumin has been shown immunologically to reach the foetus in the rabbit (Schechtman & Abraham, 1958). Whipple, Hill, Terry, Lucas & Yuile (1955) studied the transfer of ^{131}I-labelled serum proteins in rabbits and dogs when some labelled protein (unspecified) was found in the foetuses of the rabbit but not in those of the dog. Dancis & Shafran (1958) have shown that ^{131}I-labelled serum albumin, γ-globulin and β-globulin were transferred to the guinea-pig foetus. No detailed study of the comparative transmission of other homologous serum proteins to the foetus or to the liquor amnii in other animals has been reported.

The concept of the selective transmission to the foetus of particular types of antibody is now well recognized. Evidence is accumulating that

certain incomplete antibodies and antibodies with low sedimentation constants (7S) are selectively transmitted to the foetus, whereas complete blood-group antibodies and those with high sedimentation constants (19S) are not. Some heterologous antibodies cannot be demonstrated in the human new-born baby, despite the presence of a high titre in the mother. The selective transfer of types of homologous and heterologous antibodies has been studied quantitatively by immunological methods in pregnant rabbits, guinea-pigs and rats, and in suckling rats. It has been shown that there is a graded rejection of antibodies prepared in various species to the same antigen (Batty, Brambell, Hemmings & Oakley, 1954) and that these heterologous proteins may interfere with the transmission of others which normally are absorbed (Brambell, Halliday & Morris, 1958).

The anatomical membrane concerned with selective transmission in orders other than primates has been considered to be the entoderm of the gut or the yolk sac. On the basis of the evidence of all these comparative studies it has been postulated that transmission in man and other primates was by way of the amniotic fluid and the foetal gut (Brambell, Brierly, Halliday & Hemmings, 1954). The anatomy of the placental systems and foetal membranes, however, varies widely among different orders of animals. The human being possesses a specialized haemochorial placental system quite unlike that of other orders, and the yolk sac in mid and late pregnancy is entirely vestigial (Whipple *et al.* 1955). A comparable system is found only among the catarrhine (Old-World) monkeys and anthropoid primates (apes). The placenta (catarrhine) of the rhesus monkey comes to resemble the human placenta very closely in the later stages of pregnancy (Amoroso, 1952).

The experiments described here, with ^{131}I-labelled proteins injected into pregnant rhesus monkeys, provide evidence that there is a selective transfer of certain maternal serum proteins to the foetus and of others to the surrounding liquor amnii, and that the latter plays an unimportant part in their transmission to the foetus. A preliminary communication (Bangham, Hobbs & Terry, 1958) described this work.

METHODS

Animals. Six pregnant rhesus monkeys (*Macaca mulatta*) (*B*, *C*, *D*, *F*, *G*, *J*) weighing 6–8 kg were used. They were estimated to be within about 4–6 weeks of the end of their gestation periods except for monkey *G*, which was thought to be close to term (6 months). Since the exact length of gestation was not known Table 1 shows the weights of the mother and foetus and measurements of the latter at operation. No simple correlation between weight of the foetus and the estimated period of gestation was found. They had all been maintained in good condition for at least 4 months before the experiment. For 1–2 days immediately before and for the duration of the experiment they were given water containing carrier iodide (0·02% NaI) to drink *ad libitum*.

Preparation of ^{131}I-labelled serum proteins. Immediately before the monkeys were given their carrier-iodide drinking water a sample of blood was taken and the serum removed. Within 24 hr and after previous oxidation with free iodide at pH 4, 0·3 ml. of this serum was iodinated with carrier-free ^{131}I at not more than 1 atom per molecule (assuming an average molecular weight of 60,000 for all the serum proteins), according to the method of McFarlane (1958). In order to avoid risks of radiation damage the labelled serum was then diluted with 1·0 ml. of the original serum and the volume brought to 5 ml. with sterile physiological saline (SPS). The labelled serum was injected as the dose within 24 hr of iodination. The specific activity of the undiluted labelled serum was 1·6–2·0 mc in about 20 mg protein and after passage through Deacidite FF (The Permutit Co., London) it contained about 2–3% of radioactivity which was not precipitated with trichloroacetic acid (TCA).

TABLE 1. Weights and measurements of the foetuses at operation. Although the exact period of gestation was unknown there was no simple correlation of these measurements with the estimated duration of pregnancy. Rhesus monkeys, except *E* (green). Monkey *G* was considered to be very close to term

	Weight (kg)		Length (cm)	
Monkey	Mother	Foetus	Heel–cranium	Ischial tuberosity–cranium
C	7·25	—	—	—
D	7·63	0·330	31	19
E	5·2	0·400	35	21
F	6·4	0·325	27	17·5
G	6·21	0·440	28	20
J	5·8	0·480	—	—

Preparation of ^{51}Cr-labelled red cells (monkeys B, C, D, G). At the same time as blood for serum was taken, another 5 ml. of blood was taken and delivered into acid citrate dextrose (ACD) and stored in the cold during the night. The red cells were then well washed five times in SPS and finally suspended in ACD. The red cells were labelled by adding 0·8–1·0 mc of carrier-free ^{51}Cr (The Radiochemical Centre, Amersham) and allowed to stand at room temperature for 1 hr. They were then washed thoroughly with saline and kept in ACD at +4° C until they were used next day. The final activity was 30,000–40,000 counts/min/mg haemoglobin, measured at 413 mμ in a spectrophotometer.

Samples of body fluids. All samples of blood and liquor were taken into sterile paraffined syringes. Samples of urine for chromatography were taken from both mother and foetus at operation by direct needle puncture of the bladder.

Column electrophoresis of serum proteins. Serum or liquor samples measuring 0·5–1·0 ml. were subjected to electrophoresis in borate buffer $\mu = 0{\cdot}5$, pH 8·5 on columns (30 × 1·25 cm) of treated cellulose (J. H. Munktell, Sweden). The columns were developed for 8·5 hr at 600 V and the proteins then eluted in about forty 2·0 ml. fractions. Each fraction was measured for protein content and radioactivity.

Chromatographic isolation of gamma-globulin on diethylaminoethyl (DEAE) cellulose columns. Unhaemolysed samples of 1·0–3·0 ml. of serum or liquor were dialysed extensively against 0·01 and 0·02 M phosphate buffer pH 6·2 and chromatographed on columns of 1 g of DEAE cellulose. The gamma-globulins were eluted with 0·01 M buffer in 2·0 ml. fractions. Each fraction was then measured for protein content and radioactivity.

Electrophoresis of foetal gut contents. The foetus from monkey *E* was dissected within 60 min of delivery and the contents of the stomach, jejunum and ileum aspirated with syringes. The fluid from each of the latter two was mixed thoroughly with 1 ml. of carrier normal serum, centrifuged and filtered. The filtrate was then subjected to column electrophoresis as described above.

Precipitation with 10% *trichloroacetic acid (TCA).* Small portions (0·1 ml.) of samples were mixed with carrier iodide; samples with low protein concentration were also mixed with carrier serum, and then added to 10 ml. of 10% aqueous TCA. After thorough mixing the precipitate was centrifuged and a sample of the clear supernatant fluid was counted.

Measurement of protein content. Samples from chromatographic or electrophoresis columns were measured for optical density at 280 mμ in a Unicam (Cambridge Instruments) or a Uvispek (Hilger & Watts) spectrophotometer. Protein estimations on whole serum were made with biuret reagent.

Measurement of radioactivity. ^{131}I and ^{51}Cr radioactivity was measured in 2·0 or 3·0 ml. samples in a well-type scintillation counter. The efficiency for ^{131}I was 16% and the background 130 counts/min. Foetal red cells in 6 ml. of blood were washed in 6 × 30 ml. sterile normal saline containing about 1% NaI and counted as 3 ml. of packed cells.

Paper chromatography. Maternal and foetal serum and urine and liquor were loaded without pre-treatment on strips of chromatography paper 1 in. (2·5 cm) wide (Whatman No. 3 mm) and developed by ascending chromatography in *n*-butanol–acetic-acid–water (5–3–1), and in *n*-butanol–dioxane–ammonia. After 18 hr the strips were dried and placed on X-ray film for 2 weeks. From the developed radioautograph it was possible to identify the radioactive compounds by comparison with known marker spots.

Transplacental experiments

Monkeys B, C, D, G, J. The labelled serum prepared for the dose was added to the labelled red cells (monkeys *B, C, D, G*) and the solution made up to 10 ml. immediately before injecting it into the short saphenous vein. Five minutes later a sample of blood was taken from the opposite saphenous vein. From monkeys *G* and *H* a sample of blood and a sample of liquor uncontaminated by blood was withdrawn percutaneously through a No. 19 needle 21 hr after the dose. At operation 8–9 hr after the dose (monkeys *B, C* and *D*) and 24 hr after the dose (monkeys *G* and *J*), atropine was given intravenously and anaesthesia induced with thiopentone, and maintained in closed circuit with cyclopropane; monkeys *G* and *J* were maintained under anaesthesia with open ether. A mid-line incision was made through the lower segment of the uterus and the foetal membranes exposed and wiped clean and dry. From 60 to 100 ml. of liquor was aspirated through a clear avascular part of the membranes. The amniotic cavity was then entered and the foetus delivered. The umbilical cord was gently milked towards the foetus, the cord ligated and the foetus removed. The foetus was then weighed, washed in warm running water and exsanguinated by heart puncture. A sample of blood was taken from the uterine vein just before or just after delivery of the foetus. The uterus was then closed and the abdominal wall sutured.

Experiments on absorption of serum proteins from liquor amnii. ^{131}I-labelled maternal serum proteins prepared in the same way were injected percutaneously into the amniotic cavity of the conscious animal with a No. 23 gauge needle, after mixing with 3 ml. of liquor removed beforehand through the same needle. Samples of the liquor were taken at operation 8 hr later (*E*) and at 3 and 6 hr by aspiration percutaneously, and at operation 24 hr later (*F*). Caesarian section was performed as described; samples of liquor uncontaminated with blood, and of maternal and foetal blood and urine were taken at operation.

RESULTS

Transplacental experiments

Transmission of ^{51}Cr-labelled red cells

In no case was any significant amount of radioactivity measured in the foetal red cells. It was calculated that at least one part of labelled maternal red cells in three million would have been detected in the foetal red cells

counted under the conditions of the experiment. This result was taken to mean that at least no gross placental trauma had occurred and that there was no gross leakage of maternal blood to the foetal circulation.

Transmission of ^{131}I-labelled serum proteins

Qualitative analysis. A general qualitative picture of the patterns of protein and radioactivity in samples can be gained by inspection of the elution diagrams in Fig. 1. The diagram in Fig. 1*a* shows the correlation of

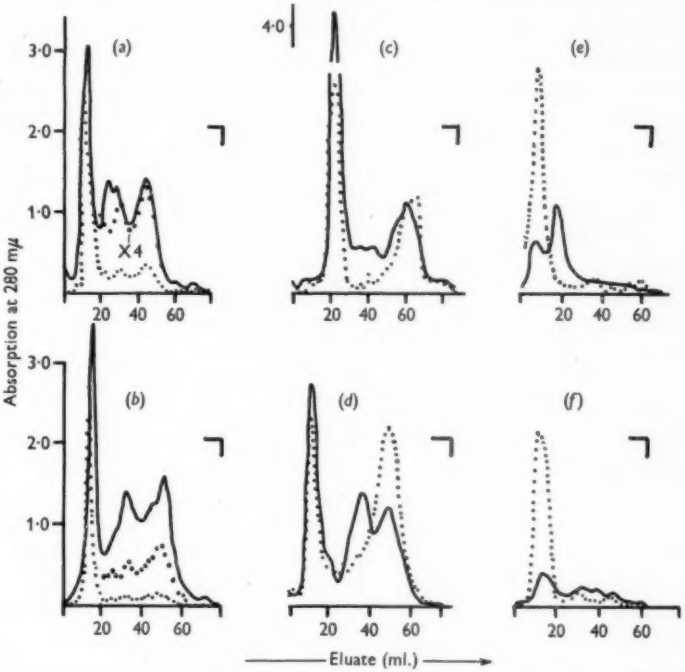


Fig. 1. Elution from electrophoresis on column of treated cellulose; in borate buffer pH 8·6, 0·1 M. —— protein measurement at 280 mμ; ..... counts/min above background (larger dots, as smaller but ×4 scale). Rhesus *D* and *G*: approximately 1·5 mc of ^{131}I-labelled maternal serum proteins injected I.V. into mother.

	Index mark (counts/min)
a, Maternal serum *G*, 1 ml. dose (diluted)	2,000
b, Maternal serum *G*, 24 hr, 1 ml.	200,000
c, Foetal serum *D*, 8 hr, 1 ml.	400
d, Foetal serum *G*, 24 hr, 1 ml.	400
e, Liquor conc. *D*, 8 hr, 1 ml.	2,000
f, Liquor conc. *G*, 24 hr, 1 ml.	2,000

radioactivity with the concentration of the serum proteins, separated by electrophoresis, in the dose. Figure 1*b* shows the change in pattern of labelled proteins 24 hr after injection intravenously into the maternal circulation. Although there are many changes in detail the over-all pattern is much the same as that of the dose. The foetal serum taken at 8 hr (Fig. 1*c*), on the other hand, shows two main peaks of radioactivity, in the albumin and γ-globulin regions. At 24 hr (Fig. 1*d*) radioactivity in the γ-globulin is increased, both absolutely and in relation to that in the albumin. Although there is radioactivity in the intervening α- and β-globulin region, particularly at 24 hr after the dose, the amount is relatively small.

The liquor amnii (Fig. 1*e* and *f*) was found to contain a large amount of radioactivity in the albumin region at both 8 and 24 hr, although the concentration of albumin is small. The over-all pattern of radioactivity is discernibly different from that of the dose of maternal serum at 24 hr, in that relatively small amounts are present in the globulin region. Furthermore, there was no difference between those monkeys which were 5 months pregnant (e.g. Fig. 1*e*) and monkey *G* (Fig. 1*f*) which was close to term, although the protein pattern had undergone a marked change due to the disappearance of a particular protein of the liquor described below. There was no peak of radioactivity corresponding to this protein.

In order to confirm that the radioactivity in the γ-globulin region behaved chromatographically like γ-globulin, dose, maternal and foetal sera and liquor were fractionated on DEAE cellulose columns. Figure 2 illustrates the elution of the various constituents of 'γ-globulin' determined in maternal and foetal sera at 24 hr in monkeys *G* and *J*. It can be seen that two major γ-globulin constituents in both monkeys contained radioactivity in both maternal and foetal serum. The second protein peak (in a displacement volume of 20–26 ml.) occurred in both maternal and foetal serum of monkey *G* (Fig. 2*a* and *c*) but in scarcely discernible quantity in the serum of monkey *J* (Fig. 2*b* and *d*). The liquor of monkey *J* was shown to contain a small amount of radioactivity and protein corresponding with the first protein but none with the second (Fig. 2*e*).

Further confirmation that electrophoretic γ_1 and γ_2 globulin were transmitted to the foetus was obtained by extending the period of time of electrophoresis before elution on a column on which 3 ml. of foetal serum had been loaded. Figure 3 shows radioactivity occurring under the two peaks of γ-globulin.

Quantitative analysis. Although the general picture may be appreciated simply by studying the figures, it is more difficult to analyse the results quantitatively. Only approximate estimations have been attempted. The total protein and radioactivity in the albumin peaks have been summed

respectively. The shapes of the peaks were fairly well defined. Where radioactivity occurred in the α-globulin region, the collecting tube on the back slope of the albumin peak showing the lowest count, or lowest protein concentration, has been included but no attempt has been made to extrapolate the albumin protein curve or the radioactivity curve down to zero. The estimation of 'total globulin' protein and radioactivity has been made by adding the values for the remaining tubes. Specific activities for albumin have been calculated by dividing the highest count contained in

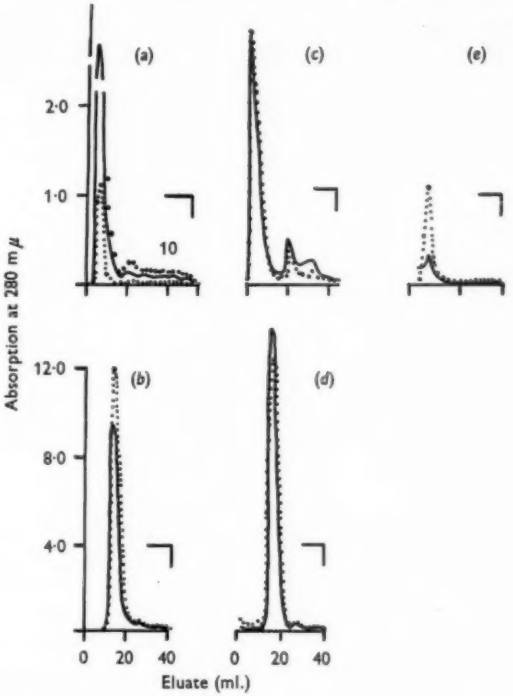


Fig. 2. DEAE cellulose column, eluted with phosphate buffer, pH 6·2, 0·02 M. —— protein measurements at 280 mμ; ..... counts/min above background (larger dots, as smaller but × 10 scale). Rhesus *G* and *J*; approximately 1·5 mc of ^{131}I-labelled maternal serum proteins injected I.V. into mother.

	Index mark (counts/min)
a, Maternal serum *G*, 24 hr, 1 ml.	50,000
b, Maternal serum *J*, 24 hr, 1 ml.	500,000
c, Foetal serum *G*, 24 hr, 1 ml.	400
d, Foetal serum *J*, 24 hr, 1 ml.	10,000
e, Liquor *G*, 24 hr, 1 ml.	400

a tube by the highest figure for optical density (protein content); this 'peak specific activity' represents that of the purest fraction of the protein obtained. Secondly, the 'total specific activity' has been calculated by dividing the total counts by the total optical density. For globulins the 'peak' figure relates to γ-globulins and it has been taken from either the clearest γ-globulin peak on electrophoresis eluates or from that of the first protein peak fraction from the chromatographic column eluate. The figures for 'total specific activity of the globulins' represent total globulin counts divided by total globulin protein. This procedure gives only a rough measure of the transmission of 'globulins' for comparison with the well-defined peak of albumin or the total of the globulins of the original serum.

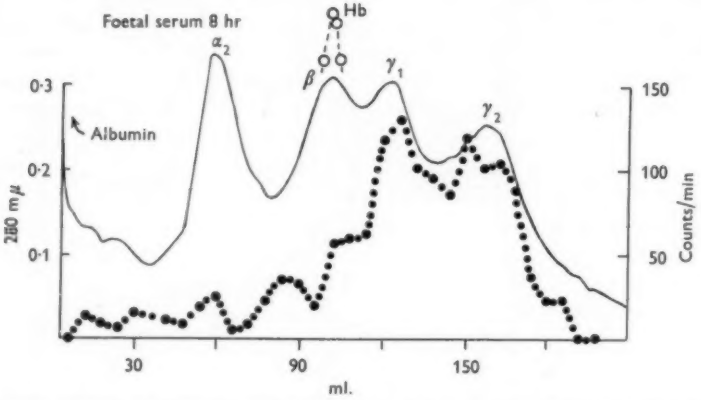


Fig. 3. Column electrophoresis as described in text. —— protein measurement at 280 mμ; ..... counts/min above background. Rhesus monkey *C*, approximately 1·6 mc of ^{131}I-labelled maternal serum proteins injected I.V. into mother. The figure shows peaks of radioactivity in the position of the γ_1 and γ_2 globulin.

Moreover, a much larger percentage of the globulin counts was contributed by γ-globulin in the foetal serum than in the maternal serum. A valid comparison of the amount of γ-globulin transmitted would be to compare the foetal concentration of γ-globulin with γ-globulin isolated from the original whole maternal serum sample. It is difficult to measure γ-globulin in whole serum in a strictly quantitative way. Complete recovery was not obtained from DEAE columns nor adequate separation on column electrophoresis; and, furthermore, it is conceivable that the radioactivity in the β- and α-regions of foetal serum may actually represent 'antibody' proteins which move electrophoretically in this region. Table 4 shows that even with these limitations clear differences in albumin:globulin count ratios occur in foetal serum and liquor compared with maternal serum.

Table 2 shows the counts per millilitre of individual samples and the approximate ratios of total albumin counts in maternal and foetal serum and amniotic fluid. The concentration in maternal serum at 24 hr after the dose is nearly half that in the sample taken at 5 min. At 21 hr (the first samples taken from monkeys *G* and *J*) the concentration in the liquor is 1/100–1/160 that in maternal serum at the same time. At 8 hr the foetal serum contained 1/545, and at 24 hr 1/200 that in the maternal serum taken at that time. The liquor thus contained about twice as much radioactive

TABLE 2. The concentrations of labelled albumin in maternal and foetal serum and in liquor at various times after labelled serum proteins had been given intravenously to the mother, and the ratios of these concentrations in these fluids

Counts/ml. of ^{131}I albumin. Dose I.V. to mother

Sample and time (hr) after dose		Monkey *D*	Monkey *G*	Monkey *J*
MS	5 min	1,044,000	—	1,424,000
	8	873,000	—	—
	21	—	356,000	1,090,000
	24	—	—	805,000
L	8	6,462	—	—
	21	—	2,120	10,800
	24	—	3,580	17,900
FS	8	1,610	1,668	4,000

Ratios of counts/ml. of ^{131}I albumin. Dose I.V. to mother

Sample and time (hr) after dose		Monkey *D*	Monkey *G*	Monkey *J*	Approximate ratios at 24 hr
MS1:MSop	8	1·19	—	—	—
	21	—	—	1·3	1–2
	24	—	—	1·76	—
MSop:L	8	140	—	—	—
	21	—	165	100	100–160
	24	—	100	(44·6)	—
MSop:FS	8	545	—	—	—
	21	—	(200)	(273)	200
	24	—	214	200	—

MS, maternal serum; MS1, maternal serum taken 5 min after the dose was given; MSop, maternal serum taken at operation (in monkey *D* at 8 hr, monkeys *G* and *J* at 24 hr); FS, foetal serum taken at operation; L, liquor.

albumin per millilitre as the foetal serum. As will be discussed later, it is possible that about half of the albumin in the foetus may have diffused out of the circulating blood volume and it appears therefore that albumin reached the foetus with probably only slightly less facility than it entered the liquor by 21 hr. Assuming the total volume of the liquor to be 100 ml. and the circulating plasma volume of the foetus to be 15 ml., the total amount of radioactive albumin in the liquor was some 5–10 times the total amount in the foetus. A rough calculation showed that about half a gram of albumin entered the liquor in 24 hr but no allowance was made for the

TABLE 3. The specific activities of albumin and γ-globulin in maternal and foetal serum and in the liquor at various times after labelled serum proteins had been given either intravenously (monkeys *D*, *G*, *J*) or percutaneously into the liquor (monkeys *E*, *F*) to the mother

Specific activity of albumin ^{131}I (counts/min/unit absorption/1 cm at 280 mμ)

		Dose I.V. to mother						Dose into liquor			
Monkey ...		*D*		*G*		*J*		*E*		*F*	
Sample and hr		Peak	Total	Peak	Total	Peak	Total	Peak	Total	Peak	Total
MS	5 min	—	—	—	—	134,000	122,000	—	—	—	—
	8	95,500	71,800	—	—	—	—	311	229	—	—
	21	—	—	—	—	85,800	77,000	—	—	—	—
	24	—	—	54,700	42,300	76,000	61,400	—	—	26	24
L	8	2,370	2,120	—	—	—	—	$2{\cdot}5\times10^6$	—	—	—
	21	—	—	—	—	16,000	12,800	—	—	—	—
	24	—	—	5,860	4,080	20,000	18,600	—	—	$2{\cdot}6\times10^6$	$2{\cdot}0\times10^6$
FS	8	96·5	112	—	—	—	—	213	170	—	—
	24	—	—	167	157	310	295	—	—	14	68

Specific activities of globulin ^{131}I (counts/min/unit absorption/1 cm at 280 mμ)

		Dose I.V. to mother						Dose into liquor			
Monkey ...		*D*		*G*		*J*		*E*		*F*	
Sample and hr		γ-peak	Total	γ-peak	Total	γ-peak	Total	γ-peak	Total	γ-peak	Total
MS	5 min	—	—	—	—	22,800	26,800	—	—	—	—
	8	11,800	11,700	—	—	—	—	—	—	—	—
	21	—	—	—	—	17,600	15,700	—	—	—	—
	24	—	—	8,970	9,500	17,700	14,500	—	—	10	—
L	8	97	150	—	—	—	—	—	—	—	—
	21	—	—	—	—	1,390	912	—	—	—	—
	24	—	—	542	515	2,380	1,180	—	—	450,000	—
FS	8	162	178	—	—	—	—	30	30	—	—
	24	—	—	373	256	990	590	—	—	45	36

MS, maternal serum; L, liquor; FS, foetal serum. The 'peak' specific activity for globulin refers to γ-globulin isolated electrophoretically or chromatographically on DEAE cellulose.

possible rate of catabolism and/or loss of albumin from the liquor in calculating this estimate.

The interpretation of the figures for the specific activities in Table 3 may be misleading, since they depend upon individual protein pool sizes as well as upon rates of diffusion. The table does, however, serve to show the high specific activity of albumin in the liquor as early as 8 hr and that by 21 hr it was only some 5–10 times lower than that in the maternal serum. Yet at the same time the concentration of albumin (in counts per millilitre) was nearly the same in the foetal serum as in the liquor.

TABLE 4. Ratio of maximum specific activities of albumin to γ-globulin in maternal serum, foetal serum and liquor

Specific activity of albumin / Specific activity of γ-globulin	Monkey *D* (8 hr)	Monkey *G* (24 hr)	Monkey *J* (24 hr)	$\frac{G+J}{2}$
Foetal serum	0·6	0·45	0·31	0·38
Liquor	24·2	11	11·5	11
Maternal serum	8·1	6	4·3	5·2

The 'specific activity' was obtained by dividing the highest counts/ml. of the albumin elution fraction (separated by electrophoresis) and the γ-globulin (isolated by chromatography), by its optical density at 280 mμ. For details, see text.

TABLE 5. General summary of findings at 24 hr

	Maternal serum	Liquor	Foetal serum
Ratio $\frac{\text{counts/ml. as albumin}}{\text{counts/ml. as globulin}}$	2–3	5	0·5
Counts/ml. as albumin	100	1	0·5
Counts/ml. as globulins	40	0·2	1
Specific activity of albumin	100	15	0·3
Specific activity of γ-globulin	20	1·5	1

^{131}I-labelled maternal serum given I.V. to pregnant Rhesus monkeys *B*, *C*, *G* and *J*. Figures of 100 % have been given to the counts/ml. as albumin and to the specific activity of albumin. The counts in the globulins in the foetal serum are largely due to γ-globulin, whereas in the liquor they are due to other globulins as well.

Table 4 compares the ratio of the maximum specific activities of albumin and γ-globulin in maternal and foetal serum and liquor while Table 5 summarizes the general quantitative findings numerically in monkeys *B*, *C*, *G*, *J* at 24 hr after intravenous injection to the mother.

Experiments on absorption of serum proteins from liquor amnii

Qualitative. Figure 4*a* shows the general pattern of distribution of radioactivity of the dose ^{131}I-labelled serum which was injected into the amniotic cavity of monkey *F*. The liquor taken at operation 24 hr later is shown in Fig. 4*b*; no significant change in the pattern of radioactivity has taken place, and its relation to the proteins of the liquor is clear. Maternal serum (Fig. 4*c*) taken at this time showed a small amount of radioactivity in the

albumin, but insignificant quantities among the globulins. The foetal serum (Fig. 4*d*) after treatment with Deacidite shows three apparent radioactive peaks, all of which are small. The results of the analysis of these samples by chromatography are seen in Fig. 5. This particular serum contained an unusually large quantity of the second main protein component to be eluted from the column under these conditions (Fig. 5*a*) which appeared to possess a much higher specific activity than the first component. Samples

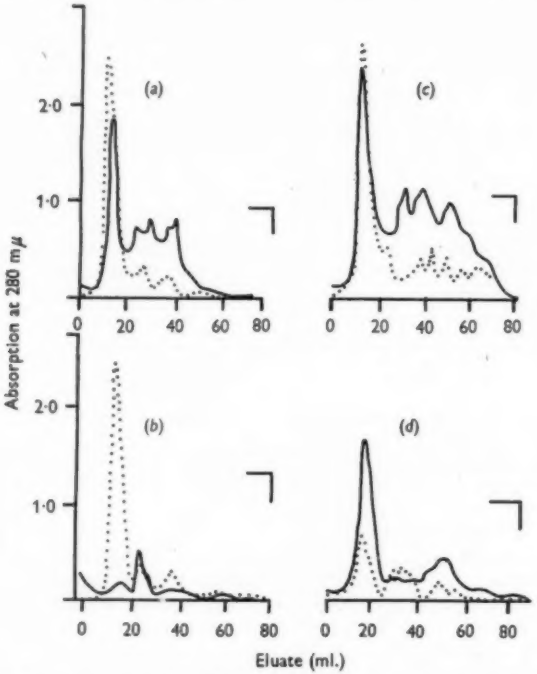


Fig. 4. Cellulose column electrophoresis as in Fig. 1. —— protein measurements at 280 mμ; ..... counts/min above background. Rhesus *F*, approximately 1·5 mc of ^{131}I-labelled maternal serum proteins injected directly into the amniotic cavity. The total amount of radioactivity injected into the liquor was the same as was injected I.V. into monkeys *C*, *D*, *G*, *J*. The specific activities of the albumin and *γ* globulin were 100 and 500 times greater, respectively, in *F*, than the specific activities of these proteins found in the liquor in monkeys *C*, *D*, *G*, *J*.

	Index mark (counts/min)
a, Maternal serum, 1 ml.	50,000
b, Liquor, 24 hr, 1 ml.	500,000
c, Maternal serum, 24 hr, 1 ml.	200
d, Foetal serum, 24 hr, 1 ml.	200

of liquor taken at 5 min, 6 hr and 24 hr showed a rapid decrease in this second radioactive component, whether chromatographed in the presence of carrier serum (Fig. 5*b* and *c*) or by itself (Fig. 5*d* and *e*). Treatment of the amniotic fluid proteins of the 24 hr sample with Deacidite did not appear to alter significantly the amount of radioactivity on the first main protein component (Fig. 5*e*), although radioautography of a paper chromatograph of the liquor at this time revealed free radioactive iodide, mono- and di-iodotyrosine and iodophenols.

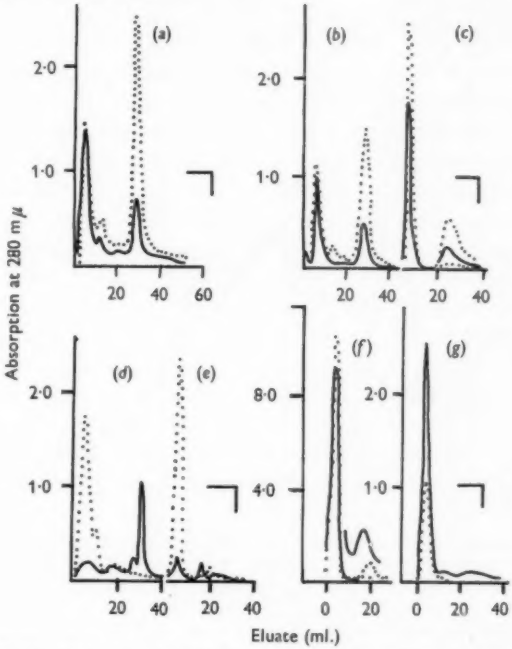


Fig. 5. DEAE cellulose as in Fig. 2. —— protein measurements at 280 mμ. ····· counts/min above background. Rhesus *F*; approximately 1·5 mc of ^{131}I. labelled maternal serum proteins injected directly into the amniotic cavity.

	Index mark (counts/min)
a, Maternal serum, 1 ml. dose (diluted)	4,000
b, Liquor, diluted with carrier serum, 5 min, 1 ml.	4,000
c, as *b*, at 6 hr, 1 ml.	4,000
d, Liquor 24 hr, 1 ml.	40,000
e, Liquor 24 hr; after treatment with Deacidite F.F.	40,000
f, Maternal serum, 24 hr, 1 ml.	100
g, Foetal serum, 24 hr, 1 ml.	100

The maternal serum (Fig. 5*f*) showed a little radioactivity in the first protein component and a trace in the second. The foetal serum showed a trace in the first component (Fig. 5*g*).

The findings on column electrophoresis of samples from monkey *E*. a green monkey approximately at term, were essentially the same.

Quantitative. The dose given to these two monkeys had the same amount of radioactivity as that used in the transplacental experiments. All of this activity was injected into the amniotic cavity. Table 3 shows that the

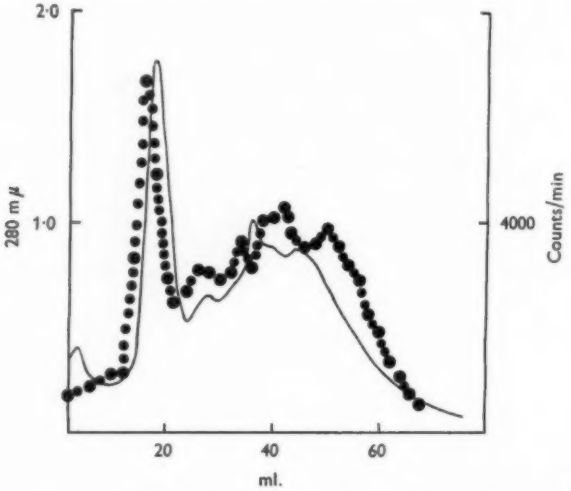


Fig. 6. Green monkey *E*; 1·5 mc of ^{131}I-labelled maternal serum proteins injected directly into amniotic cavity. The contents of the ileum of the foetus were mixed with carrier maternal serum, and a sample, after centrifugation, subjected to column electrophoresis. The similarity of the pattern of radioactivity to that in the dose serum indicates that some, at least, of the labelled proteins are present. —— protein measurement at 280 mμ; ..... counts/min above background.

specific activity of the albumin was some 100 times greater and the γ-globulin some 500 times greater than that which occurred in the liquor in the transplacental experiments. Despite this, radioactivity was found in the foetal albumin and γ-globulin in quantities which were quite insignificant compared with those found in the transplacental experiments. Slightly more radioactivity appeared in the maternal serum proteins. Since these had been diluted by the maternal circulating plasma, and to some extent by the extravascular pool, the total amount was significantly larger than that in foetal serum, but it was a small proportion of the dose within the amniotic cavity. A certain amount of this may have leaked out

through the tissue traumatized by amniotomy, and been absorbed into the maternal circulation.

To confirm that the foetus had indeed swallowed the liquor, a sample of its ileal contents diluted with carrier serum was examined, and showed a distribution of radioactivity among the proteins after electrophoresis roughly similar to that of the dose (Fig. 6).

DISCUSSION

Limitations of methods

The interpretation of these experiments depends upon the biological normality of the ^{131}I-labelled proteins. There is now reasonable evidence that proteins iodinated with an average of 1 atom per molecule behave metabolically with close similarity to proteins labelled with ^{14}C amino acids, at any rate in rabbits (Cohen, Holloway, Matthews & McFarlane, 1955). The assumption is made that the selecting mechanism of the placenta is unable to distinguish between a protein molecule so labelled and one that is not. If labelled molecules passed through the placenta then it is likely that similar but unlabelled ones would do so. Sera used for the dose in monkeys *F*, *G* and *J* were labelled with ^{131}I after oxidation of sulphydryl groups with iodine; there appeared to be no consistent gross difference in experimental results between experiments performed on these monkeys and those performed in the others.

When whole serum is subjected to the radioactive labelling and to the subsequent methods of handling and analysis it is likely that some proteins undergo denaturation more easily than others. Quantitative recovery of protein from electrophoretic or resin columns is most unusual and selective retention of particular proteins is likely to a greater or less extent. In order to control this variable, samples of serum and amniotic fluid were in many instances examined both with and without treatment on Deacidite resin columns and dialysis, before separation by column electrophoresis and chromatography. No attempt was made to preserve or to eliminate the lipo-proteins from either the dose or the samples and it is assumed that they underwent progressive denaturation with the handling of the samples.

By the methods used and in view of the small percentage of the dose of labelled proteins which was found in the foetal fluids, it was impossible to be sure whether they represented some of a protein which was present in serum in large amounts (e.g. albumin or γ-globulin) or whether they were large proportions of a protein which was present in much smaller concentration. To some extent the former was confirmed for the albumin by the fact that the electrophoretic peak was sharp and well defined and that radioactivity was found in the albumin on starch gel electrophoresis;

likewise radioactivity in the γ-globulin fractions behaved as γ-globulin on chromatography and on starch gel electrophoresis.

All monkeys were given carrier iodide in their drinking water before and throughout the experiment. Despite this, high proportions (5–30 %) of radioactivity which was not precipitable with 10 % TCA were found in foetal fluids. Whatever the physiological mechanism responsible may be, there is under these circumstances the possibility that radioactive iodide, diluted many times by the carrier iodide and iodoamino acids, may have been adsorbed on to the proteins (Block, Mandl & Keller, 1958). We have found that ^{131}I iodide is adsorbed on to Rhesus monkey albumin *in vivo* and *in vitro*. Carrier-free ^{131}I (100 μc) in saline was injected intravenously into two monkeys, one normal and one which had had carrier iodide in its drinking water for 1 week. Serum samples taken 5 min later and subjected to column electrophoresis showed radioactivity on the albumin. In serum from the normal monkey 17 %, and in serum from the monkey on carrier iodide 2·4 % of the counts per millilitre of serum were in the albumin fraction. Serum taken 10 min after the dose showed very slightly lower total counts per millilitre, and 3 and 2 % were associated with the albumin. When carrier-free ^{131}I was added *in vitro* to sera from these two monkeys, the figures were both 0·05 %. Whether true adsorption or iodination occurred or whether there was competitive adsorption between the albumin and the treated cellulose of the electrophoresis column was not explained by these data. Precipitation of the sera with TCA yielded lower percentages (about a tenth) of radioactivity associated with protein. This fact and the finding that the activity in the protein could be reduced by about 85 % by either prolonged and vigorous dialysis against repeated changes of 1 % sodium iodide in physiological saline, or by passage of the serum through a column of Deacidite, suggest that the bond is a loose one. These experiments were not designed to determine how the association of the injected ^{131}I iodide with albumin took place, nor the nature of the bond between them; they were performed to demonstrate that iodide in blood *in vivo* and in serum *in vitro* may be associated with albumin under the conditions of electrophoresis in which the experimental sera were run.

Treatment of the maternal and foetal sera and amniotic fluids on Deacidite columns and/or extensive dialysis against carrier iodide (and in one instance carrier mono-iodotyrosine) did in some samples reduce the radioactivity on the albumin peak, but only by small amounts (up to 10 %). The possibility remains, however, that a slow continuous liberation of iodide and iodoamino acids from the labelled proteins undergoing catabolism, in intimate (sometimes probably intracellular) association with serum proteins, may have resulted in a small amount of radioactivity adsorbed upon albumin in these sera.

The number of monkeys was small but the results appeared to be consistent. Monkeys *E* and *G* were within about 1 week of term, while the others were within an estimated period of 3–5 weeks of term. During the last month of pregnancy the composition of the proteins in the liquor amnii in the monkey changed markedly. At about 5 months the liquor had a total protein concentration of about one tenth of that of adult monkey serum. At this time the major protein constituent moved electrophoretically in the α_1-globulin region on the treated cellulose columns used. This protein had other characteristics which were quite different from those of α-globulins and has not yet been shown to occur in serum (D. R. Bangham, K. R. Hobbs & D. E. H. Tee, unpublished observations). A paper summarizing some of its properties is in preparation.

Samples of amniotic fluid were taken percutaneously, at 21 hr, in monkeys *G* and *J*. Although they were taken with great care, through a fine (No. 23) needle, it is possible and appears probable that some maternal serum leaked through the traumatized tissue of the needle track. This might have accounted for some of the rise in labelled albumin in the samples of fluid taken 3 hr later (particularly monkey *J*, Table 1).

Results

The experiments demonstrate two main points. First, they show a direct and selective transmission of maternal albumin and γ-globulins to the foetus, and in different proportion to the liquor amnii. Secondly, they show that the amniotic fluid plays a relatively insignificant role in the transmission of maternal serum proteins to the foetal circulation.

The quantitative observations in the transplacental experiments relate only to three individual animals at 8 and 24 hr, whereas the movement of proteins at different rates in and out of pools of different sizes represents a complex dynamic system. No data are available on the extravascular diffusion rate, metabolism or pool size of any proteins in the foetus *in utero*. The pool sizes would not change more than a few per cent during 24 hr. In some preliminary experiments we have found no great difference in the rate of catabolism of albumin in rhesus monkeys 4 weeks old compared with ones 6 months or 18 months old. The relative rates of diffusion of individual proteins through capillaries in normal animals have not been worked out. Although one may well expect each to be different, it is thought that the 'equilibrium state' will not be much different from that found at 24 hr. Nevertheless, a full picture will be obtained only by serial sampling over a period of several hours, in order to obtain a knowledge of metabolism, pool size and diffusion rates.

Transmission of proteins to the foetus. That maternal albumin is transmitted to the foetus in man has been predicted (Josephson & Gyllenswärd,

1957) on the basis of studies on changes in serum protein fraction concentrations in infants and children. Transmission of heterologous albumin has been shown by immunological methods in rabbit foetuses (Schechtman & Abraham, 1958), and homologous and heterologous ^{131}I-labelled albumin in rabbit and rat (D. R. Bangham & K. R. Hobbs, unpublished observations), and in guinea-pig foetuses (Dancis & Shafran, 1958). The evidence for the antenatal transmission of antibodies and γ-globulin in primates is considerable. Paper electrophoretic studies on serum from human foetuses from 4 to 9 months old revealed a sharp rise in the γ-globulin concentration during the last trimester (Ewerbeck & Levens, 1950). In normal infants the γ-globulin and antibody present at birth decreases exponentially for the first 3 months of life. A normal infant born to a mother with acquired agammaglobulinaemia (below 11 mg γ-globulin/100 ml. by 'immunochemical measurement') showed no γ-globulin in its serum until 3 months of age (Bridges, Condie, Zak & Good, 1959). The demonstration of the transmission of labelled γ-globulin to the foetus in monkeys is therefore largely confirmatory, with a different technique. The experiments do, however, illustrate the degree of selection which occurs in the placenta. The transmission of the γ-globulin appears so specific that it is tempting to postulate that the small amount of radioactivity present in the α- and β-globulin regions in the foetal serum represents the proteins of those antibodies known to move electrophoretically in this region. Rough estimations suggested that the total amount of radioactivity eluted from the DEAE columns accounted for about 90 % of the 'total globulin' count obtained from the electrophoresis columns. The same postulate might be made for the similar highly specific selection of labelled γ-globulin together with a small amount of labelled protein in the β-globulin region (which, however, did not correlate closely with the β-globulin peak itself) which was found in the gut of the suckling 7-day-old rat (Bangham & Terry, 1957). The method of column electrophoresis used in the transplacental experiments did not enable a more precise study of other individual proteins to be made; a few observations using starch gel electrophoresis have confirmed the general findings described.

In view of the strictly qualitative transmission of antibodies and γ-globulin it seems that the mechanism is an active one. In women suffering from toxaemia of pregnancy the concentration of α-globulin is higher, and that of albumin lower, in their sera than in normal persons; nevertheless, the new-born infant contains the same serum protein composition and concentrations as normal new-born infants (Lagercrantz, 1945). The mean γ-globulin concentrations of a group of African, European and American mothers were 1·42, 0·94 and 0·72 g/100 ml., whereas the concentrations in their new-born infants were remarkably constant, 1·03, 1·1

and 0·97, respectively (Stanier & Thompson, 1954). This situation may be compared with Halliday's finding (1957) that a suckling rat could absorb into its blood stream only a finite amount of antibody introduced into its gut. On the other hand it is possible that the increased γ-globulin in the group of African mothers was due to a high proportion of complete antibodies and/or those with a high (19S) sedimentation constant which appeared not to be transmitted to the foetus. Franklin & Kunkel (1958) have found that cord blood of normal infants contains concentrations of 19S γ-globulin much lower than in maternal blood; some 19S protein could be demonstrated by quantitative precipitin analyses, but not by ultracentrifugal analysis.

Transmission of proteins to the liquor amnii. The disputed origin of proteins in human amniotic fluid has been discussed in a recent review (Jeffcoate & Scott, 1959). In early pregnancy the liquor is similar to plasma in composition. In later pregnancy its protein composition differs from that of plasma. It has been suggested that some part at least is a product of selective secretion by the amnion, but there has been no direct experimental evidence available.

The results of the first (transplacental) group of experiments indicated that labelled maternal albumin passed readily into the liquor. At 8 hr the liquor contained about four times, and at 24 hr about twice as much per unit volume as foetal blood. It is possible that the albumin passed into both fluids with nearly equal facility and the lower concentration in the foetal blood was due to its diffusion into the extravascular albumin pool of the foetus. The total volume of the liquor was from 80 to 120 ml. and the total amount of radioactive albumin entering it was then some 5–10 times that entering the foetus. Assuming there was no loss or break-down, it was calculated that about 0·5 g of albumin passed into the liquor in 24 hr. The fate of albumin in the liquor is uncertain, but it is present in low absolute concentration. The experiments in which labelled serum was injected into the liquor suggested that it was able to return to the maternal circulation. Owing to the dilution by the maternal serum protein pool the amounts found experimentally were small, and because of the likelihood of a leak from the liquor it was considered impracticable to obtain a balance sheet for albumin entering and leaving. The proteins in the liquor may also be actively catabolized after swallowing by the foetal gut, by the foetal membranes or by enzymes within the liquor itself. Chromatography of samples of liquor from monkeys *D* and *F* showed the presence of radioactive iodoamino acids as well as iodide. Similar radioactive metabolites were found in the maternal urine, but the foetal urine contained a small amount of radioactive iodide only. No labelled or unlabelled protein was found in the foetal urine. While a measure of the rate of catabolism and

loss from the liquor was not obtained in these experiments, there was evidently a rapid turnover of albumin. The significance of this for the foetus may represent a carrier mechanism in, and perhaps out, of the liquor for small metabolically important molecules known to be carried on albumin.

The globulins on the other hand did not enter the liquor so readily; the total albumin:globulin count ratio was above that found in maternal serum and quite unlike the pattern found in foetal serum in the transplacental experiments (Table 4). It was even more difficult to obtain a reliable quantitative estimate for the globulins passing from the liquor to the maternal circulation. Since particular types of maternal antibody have been described in fairly high concentration in liquor (e.g. in Rh incompatibility) it may be that, as with the transmission to the foetus, a large proportion of a particular (but different) small group of antibodies is transmitted to the liquor while most other types are not.

The route of transmission to the foetus. The experiments indicate that both the albumin and the globulin reach the foetus via the placenta. The strongest evidence that this also occurs for antibodies in human beings is that reported by Wasz-Höckert, Wager, Hantala & Widholm (1956). They observed that the maternal-to-foetal ratio of the titre of diphtheria antitoxin in infants born with oesophageal, oesophago-tracheal, and anal atresia was the same as in normal infants. The antitoxin could therefore not have been absorbed by the gut from swallowed liquor.

In the normal non-pregnant animal serum proteins occur in other body fluids nearly always in the same relative, but often different absolute, concentrations. There are some exceptions. Joint fluid, urine, cerebrospinal fluid aqueous humour, and the secretions of several glands (saliva, pancreatic juice, seminal fluid) have been described as containing proteins different in relative concentrations and in other minor ways from those in plasma. In all these cases the quantitative discrimination is relatively small. The pregnant and suckling mammals show instances of preferential or selective transmission of one or more proteins, to a greater or less extent, where considerable quantities of proteins may be transmitted. The mammary gland of many species has been shown to select plasma γ-globulin (Askonas, Campbell, Humphrey & Work, 1954) and a small amount of plasma albumin. The selecting epithelium is ectodermal. The entodermal lining of the suckling rat selectively transmits γ-globulin but negligible quantities of other serum proteins (Bangham & Terry, 1957) and homologous antibodies in preference to heterologous ones (Batty *et al.* 1954). Much research has shown that in rats, guinea-pigs and rabbits transmission to the foetus occurs via the entodermal lining of the foetal gut and yolk-sac lining (Brambell, Hemmings & Henderson, 1951). The

absorption of antibodies from the gut in the new-born calf, a situation where non-selective absorption occurs (Bangham, Ingram, Roy, Shillam & Terry, 1958), has been shown to take place via the lymphatics of the gut (Comline, Roberts & Titchen, 1951). Whether it occurs via the lymphatics of the gut in the rat and rabbit foetus is not yet clear. There is evidence to suggest that the return of extravascular plasma proteins to the venous system occurs in most tissues in the normal animal almost exclusively via the lymphatics (Jepson, Simeone & Dobyns, 1953; Taylor, Kinmouth, Rollinson, Rotblat & Francis, 1957). There are few tissues devoid of lymphatics; these include the central nervous system, bone, striped muscle and the placentae of primates. The rhesus (and presumably also the human) foetal placenta is therefore unusual in two respects. The syncytial trophoblast is a non-entodermal membrane which selectively transmits proteins, and the proteins are transferred thereafter directly into the foetal capillaries.

The route of transmission to the liquor amnii. It is probable that the serum proteins entering the liquor do so through the surrounding chorion and amnion. It is unlikely that a significant quantity enters by way of the foetal respiratory or alimentary tract, because of the completely different specific activities and electrophoretic pattern of the labelled proteins which were found in liquor and foetal serum. No labelled protein was found in foetal urine. Apart from the relatively high concentration of the particular liquor protein which occurs in mid to late pregnancy in the rhesus monkey, human and rhesus liquor contain concentration of a tryptophan-rich 'pre-albumin' (paper electrophoresis, veronal pH 8·6, $\mu = 0{\cdot}5$) higher than that occurring in the blood. It seems, therefore, that, as in most dynamic systems, each individual protein has its own specific rate of entry into, and either break-down or exit from, the liquor. Likewise it is possible that each of these proteins has its own site (chorion, amnion, basal plate of placenta) of entry and exit, and in some cases synthesis.

Histological study of at least three membranes which are the sites of proven selective absorption of protein shows several marked similarities. Clark's studies (1959) with the electron microscope have shown with great clarity the fine structure of the entoderm of the gut of the suckling rat. The luminal surface of these cells is covered with microvilli; between the bases of the microvilli vacuoles extend into the cytoplasm of the cell. After feeding a protein antigen and taking specimens at different periods of time subsequently, he demonstrated by staining with fluorescent-labelled specific antibody that the protein was taken into the vacuoles between the microvilli by a process of pinocytosis. The many small vacuoles then coalesced as they traversed the cell to the base. The syncytial trophoblast separating the maternal blood from the foetal capillaries in

the rhesus placenta (Wislocki & Bennett, 1943) and human placenta (Wislocki & Dempsey, 1955) show similar microvilli on the surface exposed to maternal blood, and successively larger vacuoles towards the base of the syncytium. The human amnion, which has been studied by Bourne (1959), shows similar microvilli, but they occur on both the maternal and foetal surfaces, indicating perhaps the possible two-way transmission which some results of these experiments suggest. The cytoplasm in these cells in these three situations also contains many mitochondria, fat droplets and Golgi apparatus; histochemically they have been shown to contain a number of enzymes in high concentration. In general they have the appearance of being highly active.

The experiments in monkeys do not indicate whether it is the amnion or the chorion which is responsible for selective transmission to the liquor, nor whether it is that part of them which covers the surface of the placenta or that part which lines the decidua capsularis of the uterus. By analogy on a histological basis with other selecting membranes it is likely to be the amnion. If this is so, the amnion is another example of a non-entodermal membrane with this property. Furthermore, it transmits a spectrum of proteins entirely different from that transmitted by the primate placenta and rat entoderm. The amnion is also unusual in that it has no blood supply of its own and its cells depend on the viability of the foetus.

The foetal amnion, chorion (which has a maternal blood supply) and syncytial trophoblast of the placenta are unique in that they are the immunological and anatomical boundary between the genetically different maternal and foetal tissues, and yet allow certain maternal whole proteins to pass through them. Whether the placenta also allows a two-way transmission of foetal whole proteins to pass to the maternal circulation has yet to be demonstrated.

SUMMARY

1. ^{131}I-labelled maternal serum proteins were injected intravenously into pregnant rhesus monkeys. The proteins in samples of maternal and foetal serum, and liquor amnii, taken 8 and 24 hr afterwards were fractionated by column electrophoresis and by chromatography on diethylaminoethyl cellulose.

2. The foetal serum contained labelled γ_1- and γ_2-globulin and a small amount of labelled albumin. The labelled albumin was transmitted some 10–20 times less easily than γ-globulin. Since γ-globulin isolated chromatographically accounted for some 90% of the total radioactivity in the globulins, little if any of the other 'globulins' was transmitted.

3. The liquor contained about twice as much labelled albumin per unit volume as did the foetal serum. The absolute concentration of albumin in the liquor was low, and the specific activity was therefore some 40 times

that in the foetal serum. Small amounts of labelled globulins were found in the liquor and there was no preferential transmission of γ-globulin comparable with that found in foetal serum.

4. In order to determine whether proteins were absorbed from the liquor by the foetal gut, ^{131}I-labelled maternal serum proteins, with specific activities some 100–500 times higher than those occurring in the liquor in the previous experiments, were injected into the liquor of two pregnant monkeys. Twenty-four hours later extremely small quantities of labelled proteins were found in the foetal serum, and small but appreciable quantities of labelled albumin were found in the maternal serum. The fate of proteins in the liquor was not otherwise investigated, but there is apparently a high rate of turnover of albumin.

5. It was therefore concluded that the route of albumin and γ-globulins to the foetus is transplacental. The liquor plays an unimportant part, if any, in the transmission of these proteins to the foetus.

6. The histological features of the amnion and syncytial trophoblast of the placenta of the foetal monkey are closely similar to those of the entodermal lining of the yolk sac of the rabbit foetus and the entoderm of the suckling rat, sites at which selective transmission of proteins occurs. It appears probable that these two non-entodermal membranes of the foetal rhesus monkey are the sites responsible for the two instances of selective transmission observed in these experiments.

I am greatly indebted to Dr A. S. McFarlane and Mr R. C. Holloway for assistance in preparing the labelled serum; to Dr K. Sykes, Dr P. Frost and Dr D. E. H. Tee for help with anaesthesia; to Mr D. Short and Dr F. Hawking for assistance in obtaining the monkeys; and to Dr R. J. Terry with whom this work was started. It is a great pleasure to thank Mr K. R. Hobbs for his unfailing assistance throughout these experiments.

REFERENCES

Amoroso, E. C. (1952). 'Placentation'. *Marshall's Physiology of Reproduction*, Vol. 2, pp. 127–311. London: Longmans, Green and Co.

Askonas, B. A., Campbell, P. N., Humphrey, J. H. & Work, T. S. (1954). The source of antibody globulin in rabbit milk and goat colostrum. *Biochem. J.* **56**, 597–601.

Bangham, D. R. & Terry, R. J. (1957). The absorption of ^{131}I-labelled homologous and heterologous serum proteins fed orally to young rats. *Biochem. J.* **66**, 579–583.

Bangham, D. R., Hobbs, K. R. & Terry, R. J. (1958). Selective placental transfer of serum proteins in the rhesus. *Lancet*, **274**, 351–354.

Bangham, D. R., Ingram, P. L., Roy, J. H. B., Shillam, K. W. G. & Terry, R. J. (1958). The absorption of ^{131}I-labelled serum and colostral proteins from the gut of the young calf. *Proc. Roy. Soc.* B, **148**, 184–191.

Batty, I., Brambell, F. W. R., Hemmings, W. A. & Oakley, C. L. (1954). Selection of antitoxins by the foetal membranes of rabbits. *Proc. Roy. Soc.* B, **142**, 452–471.

Bergstrand, C. G. & Czar, B. (1957). Paper electrophoretic study of human foetal serum proteins with demonstration of a new protein fraction. *Scand. J. clin. Lab. Invest.* **9**, 277–286.

Bergstrand, C. G. & Czar, B. (1958). Protein bound carbohydrates in human foetal serum. *Scand. J. clin. Lab. Invest.* **10**, 379–383.

BLOCK, R. J., MANDL, R. H. & KELLER, S. (1958). The binding of mixtures of amino acids and of inorganic iodide by various serum proteins. *Arch. Biochem. Biophys.* **75**, 508–519.

BOURNE, G. L. (1959). Anatomy, physiology and pathology of the human amniotic and chorionic membranes. *Arris and Gale Lecture, Royal College of Surgeons.* London.

BRAMBELL, F. W. R. (1958). The passive immunity of the young mammal. *Biol. Rev.* **33**, 488–531.

BRAMBELL, F. W. R., BRIERLY, J., HALLIDAY, R. & HEMMINGS, W. A. (1954). Transference of passive immunity from mother to young. *Lancet*, **266**, 964–965.

BRAMBELL, F. W. R., HALLIDAY, R. & MORRIS, I. G. (1958). Interference by human and bovine serum and serum protein fractions with the absorption of antibodies by suckling rats and mice. *Proc. Roy. Soc.* B, **149**, 1–11.

BRAMBELL, F. W. R., HEMMINGS, W. A. & HENDERSON, M. (1951). *Antibodies and Embryos.* London: The Athlone Press.

BRIDGES, R. A., CONDIE, R. M., ZAK, S. J. & GOOD, R. A. (1959). The morphologic basis of antibody formation and development during the neonatal period. *J. lab. Clin. Med.* **53**, 331.

CLARK, S. L. (1959). The ingestion of proteins and colloidal materials by columnar absorptive cells of the small intestine in suckling rats and mice. *J. biophys. biochem. Cytol.* **5**, 41–50.

COHEN, S., HOLLOWAY, R. C., MATTHEWS, C. M. & MCFARLANE, A. S. (1955). Distribution and elimination of ^{131}I and ^{14}C-labelled plasma proteins in the rabbit. *Biochem. J.* **62**, 143–154.

COMLINE, R. S., ROBERTS, H. E. & TITCHEN, D. A. (1951). Route of absorption of colostrum globulin in the newborn animal. *Nature, Lond.*, **167**, 561–562.

DANCIS, J., BRAVERMAN, N. & LIND, J. (1957). Plasma protein synthesis in the human foetus and placenta. *J. clin. Invest.* **36**, 398–404.

DANCIS, J. & SHAFRAN, M. (1958). The origin of plasma proteins in the guinea-pig foetus. *J. clin. Invest.* **37**, 1093–1099.

DEUTSCH, H. F. (1954). Fetuin: The mucoprotein of foetal calf serum. *J. biol. Chem.* **208**, 669–678.

EWERBECK, M. & LEVENS, H. E. (1950). Elektrophoretische Untersuchungen am Fetalserum. *Mschr. Kinderheilk.* **98**, 436–440.

FRANKLIN, E. C. & KUNKEL, M. D. (1958). Comparative levels of high molecular weight (19S) gamma globulin in maternal and umbilical cord sera. *J. lab. Clin. Med.* **52**, 724–727.

HALLIDAY, R. (1957). The absorption of antibody from immune sera and from mixtures of sera by the gut of the young rat. *Proc. Roy. Soc.* B, **148**, 92–103.

JEFFCOATE, T. N. A. & SCOTT, J. S. (1959). Polyhydramnios and oligohydramnios. *Canad. med. Ass. J.* **80**, 77–86.

JEPSON, R. P., SIMEONE, F. A. & DOBYNS, B. M. (1953). Removal from skin of plasma protein labelled with radioactive iodine. *Amer. J. Physiol.* **175**, 443–448.

JOSEPHSON, B. & GYLLENSWÄRD, C. (1957). The development of the protein fractions and of cholesterol concentration in the serum of normal infants and children. *Scand. J. clin. Lab. Invest.* **9**, 21–38.

LAGERCRANTZ, C. (1945). Electrophoretic analysis of serum in pregnancy and in pregnancy toxaemia. *Upsala läkaref. förh.* **51**, 117–134.

MCFARLANE, A. S. (1958). Efficient trace-labelling of proteins with iodine. *Nature, Lond.*, **182**, 53.

MOORE, D. H., DUPAN, R. M. & BUXTON, C. L. (1949). Electrophoretic study of maternal foetal and infant sera. *Amer. J. Obstet. Gynec.* **57**, 312–322.

MYANT, N. B. & OSORIO, C. (1958). Thyroxine-binding protein in the serum of rabbit foetuses. *Nature, Lond.*, **182**, 866–867.

PEDERSEN, K. O. (1947). Ultracentrifugal and electrophoretic studies on fetuin. *J. phys. Chem.* **51**, 164–171.

SCHECHTMAN, A. M. & ABRAHAM, K. C. (1958). Passage of serum albumins from mother to the foetus. *Nature, Lond.*, **181**, 120–121.

SCHEINBERG, H., COOK, C. D. & MURPHY, J. A. (1954). The concentration of copper and caeruloplasmin in maternal and infant plasmas at delivery. *J. clin. Invest.* **33**, 963.

STANIER, M. W. & THOMPSON, M. D. (1954). Serum protein levels of newborn African infants. *Arch. Dis. Childh.* **29**, 110–112.

TAYLOR, G. W., KINMOUTH, J. W., ROLLINSON, E., ROTBLAST, J. & FRANCIS, G. E. (1957). Lymphatic circulation studied with radioactive plasmaprotein. *Brit. med. J.* i, 133–137.

WASZ-HÖCKERT, O., WAGER, O., HANTALA, T. & WIDHOLM, O. (1956). The transmission of antibody from mother to foetus in newborn with oesophageal atresia. *Ann. Med. exp. Fenn.* **34**, 444–446.

WHIPPLE, G. H., HILL, R. B., TERRY, R., LUCAS, F. V. & YUILE, C. L. (1955). The placenta and protein metabolism. Transfer studies using Carbon^{14}-labelled proteins in dogs. *J. exp. Med.* **101**, 617–626.

WISLOCKI, G. B. & BENNETT, H. S. (1943). The histology and cytology of the human and monkey placenta, with special reference to the trophoblast. *Amer. J. Anat.* **73**, 335–450

WISLOCKI, G. B. & DEMPSEY, E. W. (1955). Electron microscopy of the human placenta. *Anat. Rec.* **123**, 133–167.

J. Physiol. (1960), **153**, *pp.* 290–305
With 9 text-figures
Printed in Great Britain

THE MEASUREMENT OF TENSION IN VASCULAR SMOOTH MUSCLE

By A. C. BURTON and R. H. STINSON
From the Department of Biophysics, University of Western Ontario, London, Ontario, Canada

(*Received* 3 *February* 1960)

An aim of physiological research is to establish quantitative relations between physiological 'responses' and the amount of 'stimulus' that elicited them, as in terms of the concentration of a drug administered. The experimental results are most concisely and completely described, in many cases, by an equation. This may be purely empirical. When the results are examined, it may be that a logarithmic relation or a rectangular hyperbola gives a reasonable fit over the range of the variables used in the experiments. Such an empirical equation is useful over that range, for prediction of the magnitude of response from the intensity of stimulus. A familiar example is the pharmacologists' relation, found to hold over a very limited range, between logarithm of 'dose', and 'response' (Gaddum, 1953). The usefulness of such empirical equations is limited, and we cannot hope to interpret their mathematical form in terms of basic mechanisms of the response, unless that particular mathematical form is the result of some basic hypothesis, and of analysis of behaviour of a 'model' operating on this hypothesis. Since there are usually many different forms of mathematical relation that might fit the data equally well over the range, it becomes important to choose, from among these, one which might be consistent with some such hypothesis and model.

For this process to be useful, the particular response measured should obviously be 'primary' to the hypothesis and to its application to model behaviour.

An example may make this clear. Where vasomotor effects of pressor drugs are concerned, the 'primary response' might be considered to be the tension developed by the smooth muscle of the vessel wall when a given concentration of drug is presented to the 'receptor substance' of that muscle. The tension could be related to the concentration of the drug by an equation based on some plausible theory, e.g. of reversible formation of an activated complex between drug and receptor substance. The index of response measured, however, would usually be the change in resistance to

flow of a vascular bed, when a given concentration of the drug was present in the perfusate. There are three ways in which such data have been obtained. (*a*) by using perfusion at constant pressure, of an isolated vascular bed, and measurement of the decrease in flow when the perfusate contains a pressor substance in a known concentration; (*b*) by using one of the constant-flow pumps now available, again with an isolated preparation, and measuring the increase in 'driving pressure', from artery to vein, when the pressor agent is present; or (*c*) in the 'intact' circulation of an animal, where neither driving pressure nor flow are constant when the pressor agent is given.

The results of all three methods can be expressed in common terms as the change in resistance to flow (i.e. in the ratio of driving pressure to flow). As demonstrated in this paper, however, the relations between the response, in terms of change of resistance, and concentration of the drug, e.g. adrenaline, found by these three methods of experiment, will be completely different.

Figure 1, which anticipates results obtained by the methods described later in the paper, illustrates this difficulty. The data were obtained by measuring the increase in resistance to flow in the vascular bed of an isolated rabbit's ear at constant flow, repeated at several different values of that flow. Figure 1*A* shows flow–pressure curves for given concentrations of the drug. These curves closely resemble previous results obtained in this laboratory by a variety of methods (Nichol, Girling, Jerrard, Claxton & Burton, 1951; Girling, 1952). This graph contains all the data, from which we can deduce also the change of resistance that would have been measured if constant-pressure determinations had been used, instead of constant flow. Figure 1*B* shows, from the same data, the change in resistance versus concentration of drug plotted for constant pressure of perfusion, Fig. 1*C* for constant flow. Three different levels of flow and of pressure were chosen to derive these curves from Fig. 1*A* (e.g. broken lines on Fig. 1*A*). It is seen that the shapes of the resistance–concentration curves are quite different according to the method used to analyse the data. Indeed, Fig. 1*B* suggests that the response to adrenaline increases more and more as the concentration is increased, while Fig. 1*C* might suggest, for higher rates of flow, that the response was proportional to concentration. Actually, as verified in subsequent work with higher concentrations, the response eventually reaches a 'saturation' value.

Obviously we could not base the verification of any kinetic theory of action of the drug on the results of either method of experimentation, without proving which, if either, of the methods, i.e. constant-pressure or constant-flow perfusion, was related directly to the tension of the muscle. The factor that makes the curves of Figs. 1*B* and 1*C* so different is, of

course, the distensibility of the blood vessels. When constant-pressure perfusion is used, the increasing tension with higher concentrations of drug eventually causes 'critical closure' of the vessels (Burton, 1951). With constant flow, closure can never occur, since when increasing concentrations of the drug increase the tension the perfusion pressure and thus the 'transmural pressure' of the vessels also increases. This distends the vessels passively, and partially offsets the constrictive effect of the increase in

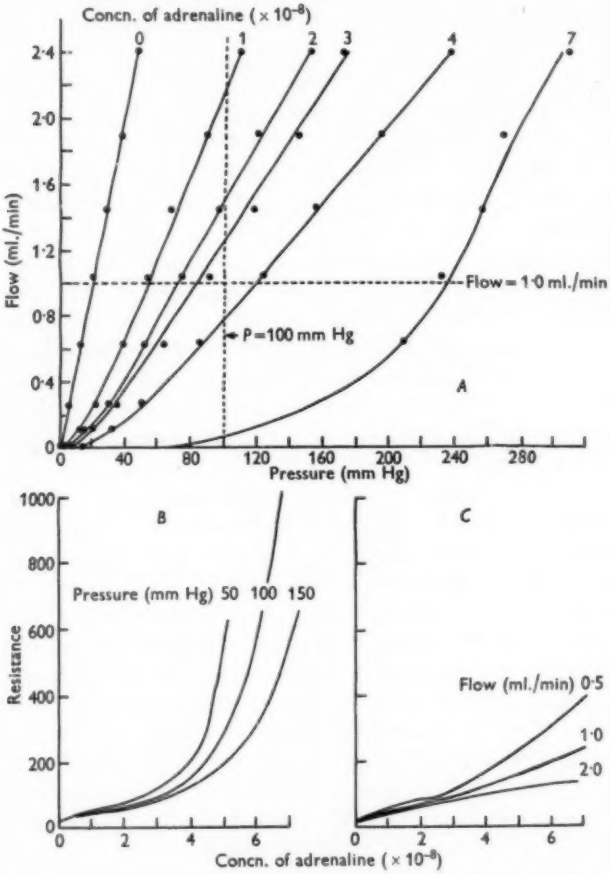


Fig. 1. *A*. Flow–pressure curves for an isolated rabbit ear, perfused with different concentrations of adrenaline. A series of values of constant flow was used.

B. Resistance to flow versus concentration of adrenaline, derived from *A*, at three different values of constant driving pressure.

C. The same for three different values of constant flow.

tension. This consideration suggested a possible method of measuring the 'active tension' of the muscle directly, in which the passive distension would be made to compensate completely for the active constriction.

Theory of a method of measuring tension of the vascular smooth muscle

To simplify the discussion we have replaced the vascular bed of the ear by a single equivalent vessel which would give the actual total resistance to flow (Fig. 2). The total tension in the wall (T), must be related to the

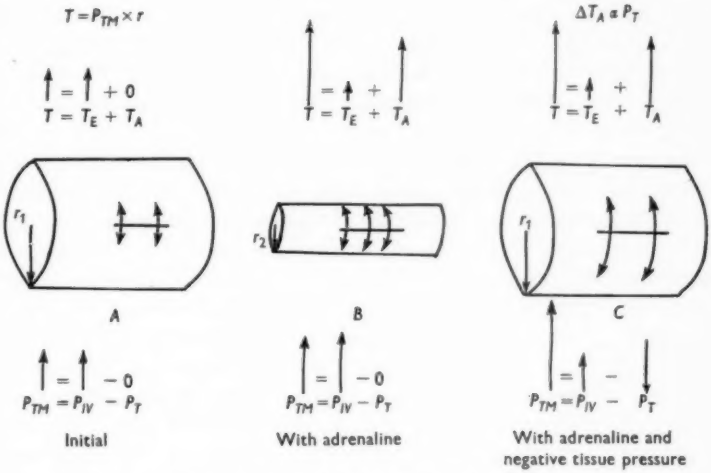


Fig. 2. To illustrate the theory of the 'null method' for measuring active tension in the vessel wall.

'transmural pressure' (P_{TM}), which is the intravascular pressure P_{IV} minus the tissue pressure P_T, by the law of Laplace (*ca.* 1806), $T = P_{TM} \times r$, where r is the radius of the equivalent vessel (Burton, 1951). The total tension T can be considered as the sum of two tensions: the 'elastic tension', T_E, which depends on the degree of stretch of the wall, plus the 'active tension', T_A, due to contraction of the smooth muscle.

By our definition, 'active tension' is independent of the degree of stretch of the wall. Vascular smooth muscle, the contraction of which supplies the 'active tension', also possesses 'elasticity', i.e. change of tension with stretch, but its contribution in this respect can be included under the 'elastic tension', most of which is probably due to the other elements of the wall (Burton, 1954). Thus the division into 'active' and 'elastic' tensions is 'functional' and convenient, and not on an anatomical basis, nor does it involve any approximation. Without the presence of the vasoactive drug,

T_A may be considered to be zero, as in Fig. 2*A* (the result does not depend on this assumption).

When the drug is present in a given concentration, the active tension increases, and the vessel contracts, as in Fig. 2*B*. The total tension has increased. However, the increase in active tension T_A has been partially offset by a decrease in the elastic tension, T_E, due to the reduction in stretch of the wall. Thus the change in total tension cannot be used as an index of the primary response, i.e. of the active tension, unless the details of the concomitant decrease in the elastic tension are known. This would involve knowing the details of the distensibility curves of the resistance vessels.

However, if the vessel can be made to return to its original radius in the presence of the pressor drug, then the elastic tension will also return to its original value (Fig. 2*C*). It is possible that this assumption may not be correct, for there is evidence that the contraction of the smooth muscle may 'tighten up' the elastic fibres of the wall (see Appendix) so that even though the radius is the same as before, the elastic tension may be greater. Making this assumption is the best that can be done. The increase in total tension will now equal the change in active tension induced by the drug, i.e. $\Delta T = \Delta T_A$. Since the radius of the vessel depends on the transmural pressure, P_{TM}, which is the difference between intravascular and tissue pressure ($P_{TM} = P_{IV} - P_T$), the vessel may be distended to its original size by decreasing the tissue pressure, i.e. by lowering the pressure in a box in which the rabbit's ear is placed (Fig. 3). If a 'negative pressure' sufficient to reduce the driving pressure to its original pressure be found, and the flow is constant, the resistance to flow must be once more at its original value. Since the resistance to flow depends on the radius of the vessel, this must also be at its original value, as was desired (except for the reservation below).

The law of Laplace is

$$T = P_{TM} \times r.$$

If r has been kept constant by the procedure above, then

$$\Delta T_A = \Delta T = \Delta P_{TM} \times r,$$

and since the intravascular pressure will be the same as before

$$\Delta P_{TM} = -\Delta P_T.$$

Thus, in this 'null method',

$$\Delta T_A = -r(\Delta P_T).$$

Since the parameter r in this null method is a constant, the negative tissue pressure required to reach the null point will therefore be a measure of the active tension produced by the drug in the vascular smooth muscle.

This analysis is, of course, based on a 'lumped parameter', the 'single equivalent vessel', and in the real situation several categories of vessel contribute to the total resistance to flow. Since distensibilities of different categories of vessel are not the same, the final situation when negative tissue pressure is applied might give the same total resistance, yet the arterioles might not be exactly at their original radii, because other vessels contributing to the resistance were now distended. This statistical consideration is a valid criticism of the method, but the method is the best that can be devised. The error is unlikely to be great, since there is abundant evidence, from calculations based on the sizes of vessels, and from the pressure gradients down the line of resistances in vascular beds, that one category of vessels, the arterioles, contributes by far the greatest part of the total resistance (probably at least 70%), especially when there is vasomotor tone.

The null method, which employs negative tissue pressure, is not very convenient, and cannot be used for long periods of time, e.g. to establish a large number of tension–concentration relations, because oedema of the ear occurs quite rapidly as a result of the increased capillary transmural pressures. However, this method provides a means of testing measures more easily available, such as the increase of resistance produced by the drug when either constant-pressure or constant-flow perfusion is used, as to their relation to the active tension. Fortunately it turns out that the rise of resistance, at constant flow, is an excellent measure of active tension.

METHODS

Rabbits, weighing 1·8–3 kg, were anaesthetized with urethane (7·5 ml./kg of a 20% solution, intraperitoneally). Heparin (2 mg) was injected intravenously. The central and the major peripheral veins of the ear were first cannulated with polyethylene tubes and then the central artery near the root of the ear. The blood was forced out of the ear by a syringe, and the ear was then cut off. In the 'null method' it was essential that venous outflow, other than through the cannulae, should be occluded, since otherwise the effective venous pressure might be indeterminate. A paraffin-wax plug was poured into the auricular cavity at the root of the ear, and a tight ligature was tied over the skin, holding it against this plug. A coat of collodion was also applied to seal off any seepage of blood. In spite of these precautions, leaking sometimes occurred when high concentrations of adrenaline were used, necessitating very high values of the negative pressure to reach the null point. Leakage was easily detected, and where it occurred the data were rejected.

All the plastic tubes of the cannulae, arterial and venous, were led through a glass tube in a rubber stopper in the wall of the chamber. The venous cannulae drained into a test-tube, to preserve a constant level of venous pressure. The artery was connected through a constant-flow pump (sigmamotor) to a funnel containing a known volume of buffered Ringer's solution, at pH 7·4. This solution was prepared by mixing two stock solutions which were stored at 4° C until required. One part of a solution of $CaCl_2$ (2·16 g/l.) was added to 8 parts of a solution of 76·5 g NaCl, 3·78 g KCl, 4·50 g anhydrous Na_2HPO_4, and 1·5 to 2·5 ml. M-H_3PO_4 to pH 7·4 in 8 l. of water.

The total volume of fluid in the system could be reduced to less than 5 ml. of which only 0·1–0·2 ml. was in the vessels of the ear. The pump delivers a pulsatile flow, the mean value of which is accurately known from the calibration of a continuously variable gear on its motor. The output is completely independent of the resistance to flow into which it pumps.

The driving pressure was measured by a strain-gauge electromanometer (Statham) connected to the arterial cannula and was recorded on an ink-writing recorder (Varian).

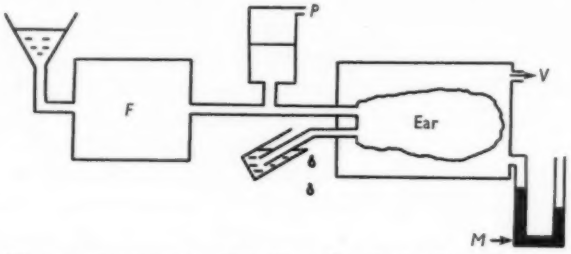


Fig. 3. Diagram of apparatus used in the null method. *F*, constant-flow pump; *M*, manometer showing negative tissue pressure; *P*, pressure transducer for driving pressure; *V*, vacuum pump.

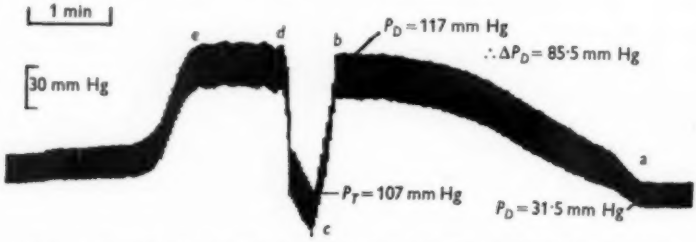


Fig. 4. Record obtained by the 'null method'. For explanation, see text.

Solutions of fresh adrenaline (Parke, Davis and Co; epinephrine hydrochloride solution C.S.D. 1/100) were made up in concentrated form (1 in 10^5 or 1 in 10^6) at pH 4·0, in which the *in vitro* oxidation is inhibited. Small volumes of these solutions were added to the funnel of Ringer's solution, which was quickly stirred to give the various concentrations of drug desired. It was found early in the research that the addition of as little as 1 part in 200 of blood or plasma (or of a 0·8 mg % glutathione solution) to the perfusate removed any evidence of *in vitro* oxidation of the drug during its passage through the ear, and made the concentration–response curves much more consistent, as well as increasing the responsiveness.

As has been general experience, the isolated ears, perfused by cold Ringer's solution, were responsive to concentrations of adrenaline as low as 10^{-9} g/ml. Oxygenation of the perfusing solution by bubbling O_2 in the funnel made no appreciable difference to this responsiveness. There was very little change in responsiveness in the 1–2 hr period of an experiment.

Operation of the 'null method' for measuring the tension

Figure 4 is a typical record from an experiment at a constant flow of 2·5 ml./min. At the point marked '*a*', the reservoir funnel was emptied and the solution was replaced by Ringer's solution containing a concentration of 2×10^{-8} of adrenaline. When the subsequent

rise of pressure reached a steady level, at point '*b*', the pressure in the chamber was reduced (by a vacuum pump) until the driving pressure was below the original value, point '*c*'. As soon as the box pressure was lowered the venous outflow ceased, and some fluid from the outflow test-tube was observed to flow back into the ear to fill the distended vessels. For up to 30 sec, the record of driving pressure showed a rise as the new steady state of flow was reached. Air was then allowed to leak into the chamber until the mean driving pressure

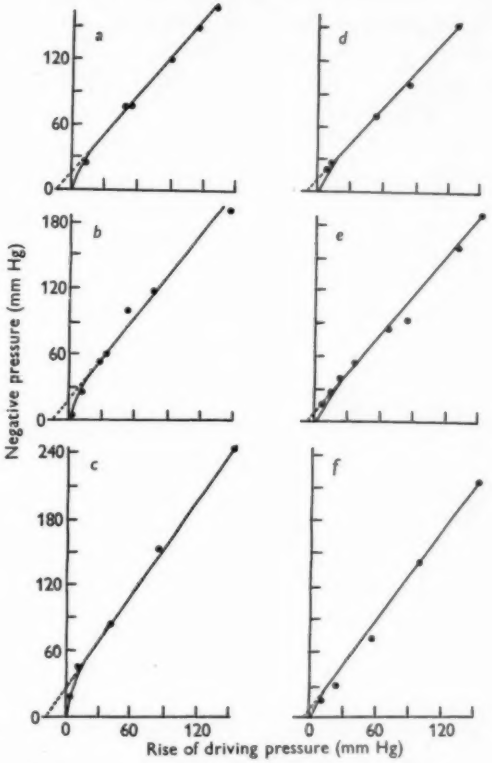


Fig. 5. Relation of negative pressure in the chamber to rise of driving pressure at constant flow for six different ears.

reached its original value. The pressure in the chamber was rapidly returned to atmospheric, and the driving pressure rose to the original value (point '*d*'). Finally (point '*e*') the solution in the funnel was replaced by fresh Ringer's solution, and the driving pressure returned to its original value. The measurements of the records, and of a mercury manometer attached to the chamber, gave the simultaneous values of the rises of driving pressure P_D at constant flow, and of the increased transmural pressure (the negative pressure in the chamber) required to bring the vessels back to their original resistance.

RESULTS

The graphs of the negative tissue pressure, for the 'null' point, proportional by the theory to the tension of the smooth muscle, versus the rise in driving pressure for six different ears, are shown in Fig. 5. Graphs *a*, *b*, *c* and *d* are at the same constant-flow rate; *e* and *f* are at two other rates of flow. It may be seen that if the drug produces rises of pressure of

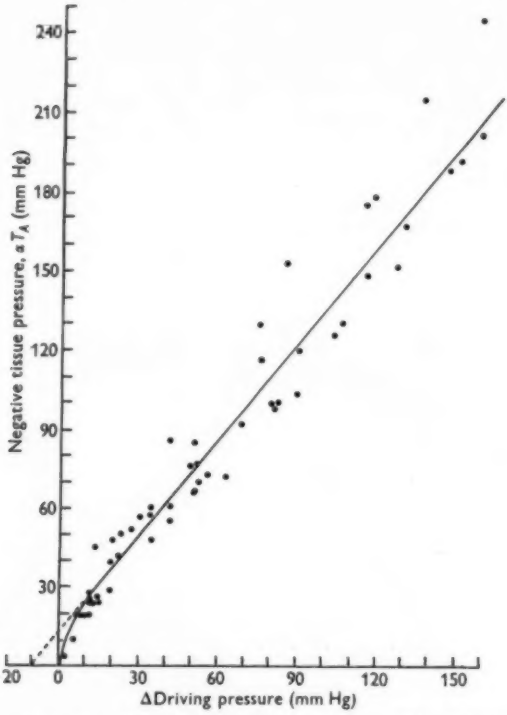


Fig. 6. Results for ten different ears, each at a constant flow of 2·52 ml./min. The coefficient of linear correlation for abscissae greater than 20 mm Hg is 0·96. $-P_T = 1{\cdot}26P_D + 12$, $\therefore T_A \propto (\Delta P_D + 10)$ for $\Delta P_D > 20$.

more than 20 mm Hg the relation is remarkably linear. The straight line, when extrapolated, has an intercept of about minus 10 mm Hg, for the flow rate of 2·5 ml./min. In this range, therefore, the tension of the smooth muscle is accurately proportional to the rise of arterial pressure plus a small constant added pressure (10 mm Hg in this case). In the case of the other rates of flow used, the added constant is less, and could be neglected.

The results of many experiments at the intermediate flow rate of 2·5 ml./min on ten different ears are shown on a scatter graph in Fig. 6. The linearity for pressure rises of more than 20 mm Hg is evident even when many ears are included. The coefficient of correlation was 0·96 (37 degrees of freedom). Similar scatter graphs were made for flow rates of 1·72 and 3·50 ml./min. The coefficients of correlation were again very high, 0·98 (16 degrees of freedom) and 0·97 (14 degrees of freedom) respectively. The slope of the regression line for the flow rate of 1·72 ml./min was significantly different from that of the data at a flow rate of 2·52 ml./min, and in the case of the highest and lowest flow rates the intercepts of the regression lines on the pressure axis were between 0 and -5 mm Hg, which would be negligible for practical purposes.

DISCUSSION

It is evident that when the method of perfusion at constant flow is used, the increase in driving pressure (or in the proportional resistance to flow) is a very good measure of the increase in 'active tension' of the vascular smooth muscle when a given concentration of adrenaline is present in the perfusate. There is very little error in assuming strict proportionality between the rise in driving pressure and the active tension. Greater accuracy for the flow rate of 2·5 ml./min used in the majority of the experiments is achieved by adding a constant 10 mm Hg to the observed increase of pressure, for pressure increases greater than 20 mm Hg. Proportionately less should be added for increases of less than 20 mm Hg, say 3 mm Hg for ΔP_D between 0 and 10 mm Hg, and 7 mm Hg for ΔP_D between 10 and 20 mm Hg. For the two other flow rates (1·72 and 3·50 ml./min) no correction is necessary.

Since the concentration–response curves when the experiments are made with constant perfusion pressure, rather than with constant flow, are completely different, it is obvious that the constant-pressure method offers no possibility of measuring the 'primary response'.

The linearity of the relation with 'active tension' in the constant-flow method is not to be thought of as arising from any simple basic relations, but as an 'accidental' result of many factors concerned with the vascular resistance. The degree of distensibility (change of radius with transmural pressure) is one of these factors, the fourth power relation between radius of vessel and viscous resistance is another, and mathematical analysis (see Appendix) indicates that a decrease in distensibility when the smooth muscle contracts is also involved. It should be emphasized, therefore, that this fortunate linearity in the case of the rabbit's ear, at the rates of flow employed, must not be taken as an indication that a similar linearity

would exist in other vascular beds under different circumstances of pressure and flow. A generalization that the method of constant flow would be more likely to give such linearity than the more common method of perfusion at constant pressure would, however, be justified. The mathematical analysis given in the appendix encourages the hope that approximate linearity would be found in other vascular beds.

SUMMARY

1. The need is pointed out for a method of measuring the 'active tension' of vascular smooth muscle in response to a given concentration of a pressor drug, or to nervous stimulation, if interpretation in terms of kinetics of drug action is to proceed.

2. It has been shown that if the 'response' is measured by a change of resistance to flow of the vascular bed, the relation between response and concentration of adrenaline is quite different if constant-pressure perfusion is used, from the results with perfusion at constant flow.

3. A 'null method' for measuring the 'active tension' directly has been devised, based on a 'lumped parameter' theory of a single equivalent vessel having the same resistance to flow as the vascular bed.

4. When the results of this 'null method' are compared with the rise in driving pressure obtained under constant flow, in a range of concentrations of adrenaline from threshold concentrations to those giving maximal response, it is found that the relation is linear for all but the smallest responses (increases in driving pressure of less than 20 mm Hg). In contrast, the changes of resistance observed when constant perfusion pressure is used are not at all linearly related to the active tension.

5. It is concluded that for the rabbit ear preparation the measurement of responses to adrenaline as increases of driving pressure, or of resistance, at constant flow should permit interpretation in terms of basic theories of action of the drug on the smooth muscle.

The work was made possible by the granting of leave of absence to one of us (R.H.S.) from Ontario Agricultural College, Department of Physics. We are grateful to Mrs D. Elston for technical assistance. This research was supported by a grant of the Medical Division of the National Research Council of Canada.

APPENDIX

Theory of constant pressure and of constant flow perfusion

The concept of a 'single equivalent resistance vessel' is used, as already explained in the text. The total tension, T, per centimetre length of vessel, is given by

$$T = T_E + T_A, \tag{1}$$

in which the 'elastic tension', T_E, is a function of the radius of the vessel (Fig. 7); i.e.

$$T_A = T - f(r). \tag{2}$$

For the total tension T we have the law of Laplace,

$$T = P_{TM} \times r, \tag{3}$$

where P_{TM} is the transmural pressure of the single equivalent vessel.

It is easily shown that if the use of the 'single equivalent vessel' is justified, i.e. most of the total resistance to flow resides in one set of vessels (the arterioles), then the mean intravascular pressure at the single equivalent vessel must be midway between arterial and venous pressures. If venous pressure be taken as zero, then

$$P_{TM} = P_D/2, \tag{4}$$

where P_D is the 'driving pressure', arterial minus venous pressure. By substitution, equation (2) becomes

$$T_A = \frac{P_D . r}{2} - f(r). \tag{5}$$

Equation (5) applies whether constant-pressure or constant-flow methods of perfusion are employed.

Perfusion at constant driving pressure

Here P_D is constant in equation (5). A graphical solution of the equation has been given elsewhere (Burton, 1951), and is repeated in Fig. 7*A*. The curve represents the rise of elastic tension with increase of the radius r of the vessel, beyond its unstretched radius r_0. The straight line (Laplacian line) through the origin, with slope equal to $P_D/2$, represents the total tension that must be present if the law of Laplace is to be obeyed. The intersection at point A represents the radius and elastic tension of the vessel when it is in equilibrium under elastic tension only, without any vasomotor tone ($T_A = 0$). Suppose that under vasomotor tone the vessel contracts to radius r', then the new total tension is represented by the vertical distance BD. Of this, CD represents the now reduced elastic tension. The remainder, BC, must represent the active tension T_A required to reduce the radius from r to r'. On the basis of this diagram the possibility of 'critical closure' was predicted, since when the intercept BC has reached its maximum value ($B'C'$), at a value of r close to r_0, no greater active tension than this will be required to cause complete closure of the vessel, unless new forces intervene.

Elastic diagrams of blood vessels (Roach & Burton, 1957) are such that at a moderate degree of stretch beyond the 'unstretched radius' r_0, the

line of elastic tension reaches its maximum slope, since all the collagenous fibres in the wall are now being stretched. Except for the lowest values of active tension and of transmural pressure, therefore, we may use an approximation for $f(r)$,

$$f(r) = k(r-r_0), \tag{6}$$

where k is the maximum elastic modulus of the wall. Substituting this in equation (5) yields

$$T_A = kr_0-(k-P_D/2)r,$$

$$\therefore \quad r = \frac{kr_0-T_A}{k-P_D/2} \tag{7}$$

(k must be greater at $P_D/2$, as inspection of the diagram will show; otherwise there will be no intersection, indicating 'blow-out' of the vessel under too high a pressure).

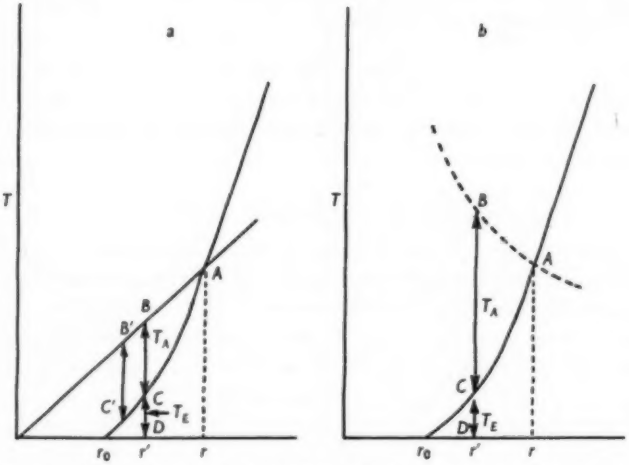


Fig. 7. Illustrating the equilibrium of the single equivalent resistance vessel. *a*, with perfusion at constant pressure; *b*, with perfusion at constant flow.

We may deduce how the resistance to flow will depend on the active tension, T_A, by using the Poiseuille–Hagen formula,

$$R = \alpha r^{-4}, \quad \text{where} \quad \alpha = \frac{8\eta l}{\pi}. \tag{8}$$

Finally,

$$R = \frac{\alpha(k-P_D/2)^4}{(kr_0-T_A)^4}. \tag{9}$$

Equation (9) predicts a very accelerated rate of increase of resistance as T_A increases, because the fourth power is involved, until finally, when

$T_A = P_D/2 \times r_0$, the point of critical closure is reached. Such a very curvilinear relation between resistance and concentration of adrenaline (related to T_A) was found experimentally (Fig. 1*B*) when constant-pressure perfusion was used.

Perfusion at constant flow

Equation (5) applies, but P_D is no longer constant. Instead,

$$P_D = F \times R = \alpha F r^{-4}, \tag{10}$$

where F is the constant-flow rate. Substituting in equation (5),

$$T_A = \frac{\alpha F}{2} r^{-3} - f(r). \tag{11}$$

The solution of equation (11) is represented graphically in Fig. 7*B*, where the broken line represents the first term (in r^{-3}). The intercept BC is now much greater than in Fig. 7*a*, i.e. much more active tension is required to reduce the radius of the vessel. This is, of course, because of the compensatory rise of transmural pressure that occurs in the constant-flow method. There is now no maximum value of the intercept $B'C'$ (as in Fig. 7*a*), and so no possibility of 'critical closure'.

Again we may use the approximation for $f(r)$, equation (6), and find

$$T_A = kr_0 + \frac{\alpha^{\frac{1}{4}} F R^{\frac{1}{4}}}{2} - k\alpha^{-\frac{1}{4}} R^{-\frac{1}{4}}. \tag{12}$$

How can this equation represent an almost linear relation, except for very small values of T_A, between T_A and the resistance R? (The driving pressure P_D plotted in Fig. 5 is proportional to the resistance R if the flow is constant.) In a plot of T_A versus R, the term $R^{\frac{1}{4}}$ will give a relatively slight curvature towards the axis of R over the whole range. The term in $R^{-\frac{1}{4}}$, which is smaller, decreases rapidly as R is increased. Subtraction of this term from the other will tend to straighten the line, over the range in which the term is not negligible. To illustrate this, numerical calculation has been made of the function

$$y = nR^{\frac{1}{4}} - R^{\frac{1}{4}}$$

with n having values of 1, 2, 3 and 4 (Fig. 8).

The working range of the experiments at constant flow corresponds to an increase of the resistance of, at the most, five times, as the maximal effect of adrenaline is reached before this. The linearity and correspondence to the experimental curves is moderately good. There is, however, evidence of a further factor that makes the linearity more marked. This is the evidence that when there is vasomotor tone the value of k increases.

The sigmamotor pump supplies a constant fluctuation in volume flow, which is shown by the fluctuation in driving pressure on the records, i.e. the 'pulse pressure'. The records by the 'null method' for the active tension (e.g. Fig. 4) show that, even when the vessels are brought back to their original size, the pulse pressure increases with the concentration of adrenaline. This indicates an increase in the elastic modulus, k, brought about by the 'tightening-up' of the 'slack' in the collagenous fibres when

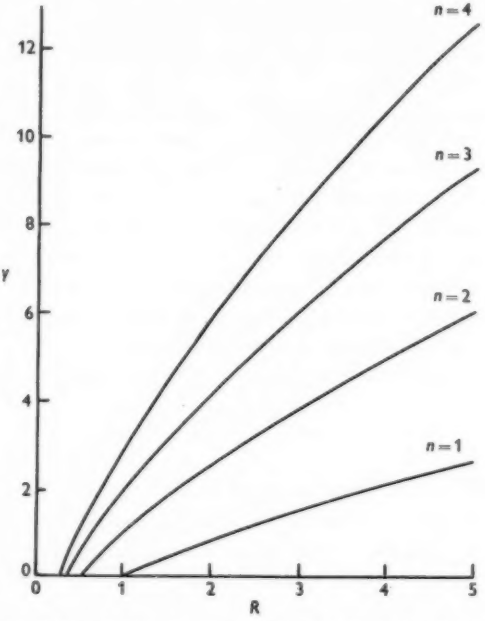


Fig. 8. Plot of the function $y = nR^{\frac{1}{2}} - R^{-\frac{1}{2}}$ for values of n of 1, 2, 3 and 4.

the smooth muscle contracts. There is an increase in pulse pressure to about twice its original value as the resistance increases three times (Fig. 9B). We therefore corrected the numerical calculation to

$$y = nR^{\frac{1}{2}} - f(R^{-\frac{1}{2}}),$$

where f increased from 0·5 to 1·0, as indicated in Fig. 9A. The result is a marked straightening of the line, except for the lowest values of T_A. It now resembles the experimentally determined curves of Fig. 5 very closely indeed.

It is concluded, therefore, that the remarkable linear relation found experimentally, with perfusion at constant flow, is consistent with the

theory, and that the same theory predicts that the method of perfusion at constant driving pressure would give no such convenient linear relation between active tension and response. While it is not at all conclusive, the theory does offer hope that other vascular beds than that of the isolated rabbit's ear might obey similar linear relations if the method of constant flow were employed.

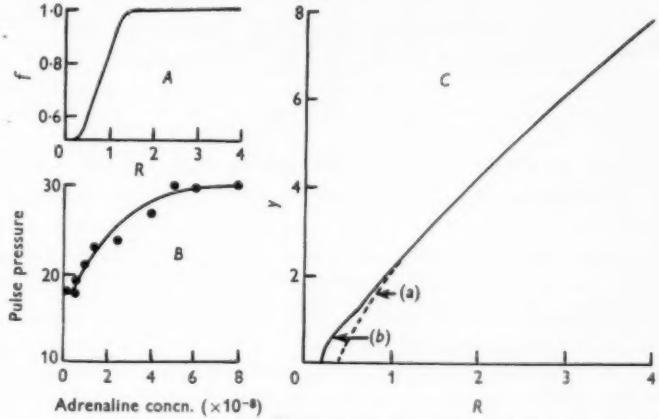


Fig. 9. *A*. Assumed variation of the factor f in the relation $y = nR^{\frac{1}{2}} - fR^{-\frac{1}{2}}$. *B*. Experimental results for pulse pressure versus concentration of adrenaline. The pulse pressure was measured at the 'null point', i.e. with the single equivalent vessel at the same size. *C*. Application of the correcting factor f to the curve of Fig. 8 ($n = 3$). (*a*) broken line, uncorrected ($y = 3R^{\frac{1}{2}} - R^{-\frac{1}{2}}$; (*b*) solid line, corrected, ($y = 3R^{\frac{1}{2}} - fR^{-\frac{1}{2}}$).

REFERENCES

Burton, A. C. (1951). The physical equilibrium of the small blood vessels. *Amer. J. Physiol.* **164**, 319–329.

Burton, A. C. (1954). Relation of structure to function of the tissue of the wall of blood vessels. *Physiol. Rev.* **34**, 619–642.

Gaddum, J. H. (1953). Bioassays and mathematics. *Pharm. Rev.* **5**, 87–134.

Girling, F. (1952). Vasomotor effects of electrical stimulation. *Amer. J. Physiol.* **170**, 131–135.

Laplace, P. S. (*ca.* 1806). In a supplement to the tenth book of *Mécanique Céleste*, cited in Encyclopaedia Britannica, Vol. 21, p. 594, 1957 ed. Article originally by Maxwell, J. C., modified by the third Lord Rayleigh.

Nichol, J. T., Girling, F., Jerrard, W., Claxton, E. B. & Burton, A. C. (1951). Fundamental instability of the small blood vessels and critical closing pressures in vascular beds. *Amer. J. Physiol.* **164**, 330–344.

Roach, M. & Burton, A. C. (1957). The reason for the shape of the distensibility curves of arteries. *Canad. J. Biochem. Physiol.* **35**, 681–690.

J. Physiol. (1960), **153** *pp.* 306–330
With 13 *text-figures*
Printed in Great Britain

SYNAPTIC LINKAGE BETWEEN AFFERENT FIBRES OF THE CAT'S HIND LIMB AND ASCENDING FIBRES IN THE DORSOLATERAL FUNICULUS

BY A. K. McINTYRE AND R. F. MARK

From the Department of Physiology, Medical School, University of Otago, Dunedin, New Zealand

(*Received* 12 *February* 1960)

In contrast with the very large amount of work carried out on the synaptic mechanisms of motoneurones, relatively few investigations have been made into the properties of the synapses which interrupt afferent pathways, although the latter junctions have long been known to possess distinctive properties (Marshall, 1941; Therman, 1941; Grundfest & Campbell, 1942; Lloyd & McIntyre, 1950; Amassian, 1953; Bishop, 1953; Rose & Mountcastle, 1954). Of the afferent junctional systems previously studied, the linkage between hind-limb primary afferent fibres and neurones projecting rostrally in the ipsilateral dorsolateral funiculus is of special interest because low-threshold (Group I) afferent fibres serving muscle effect monosynaptic connexions with neurones of this projection system (Clarke's column cells) as well as with motoneurones (Lloyd & McIntyre, 1950). Thus a favourable situation is provided for comparing the properties of an afferent with those of the well-known motoneurone synaptic system. The object of the experiments to be described was to explore more fully the differences between these two synaptic systems, and to examine the response patterns evoked in single dorso-lateral tract neurones by different forms of peripheral stimulation. Many of our results are in substantial agreement with those of Laporte, Lundberg & Oscarsson (1956*a*, *b*) and Laporte & Lundberg (1956), which became available when this investigation was at an advanced stage. Some of our findings have already been reported briefly (McIntyre, Mark & Steiner, 1956; Mark & McIntyre, 1956).

METHODS

Adult cats were used in all experiments, usually anaesthetized with pentobarbitone (Nembutal; Abbott Laboratories) in initial dosage of 40 mg/kg given intraperitoneally. Anaesthesia was maintained by intravenous administration of additional pentobarbitone as required. In a few experiments the animals were used in the acutely decapitate state after initial ether anaesthesia. Laminectomy was performed in order to expose the spinal cord from the lumbosacral to the upper thoracic segments; rigid fixation of the vertebral column and pelvis was secured by a series of clamps attached to a heavy base-plate. In

some experiments, electrical activity of the dorsolateral tract was recorded in volume by a surface lead, as in a previous study (Lloyd & McIntyre, 1950); in others, monophasic records of the tract discharge were made from a length of the dorsolateral funiculus in continuity caudally, but severed rostrally and dissected from the cord for several centimetres. This method was suggested by the work of Rudin & Eisenman (1951), and has been used extensively by Lundberg and his colleagues (Laporte *et al.* 1956*a*; Oscarsson, 1956, 1958). Nerves or spinal roots pertaining to the ipsilateral hind limb were dissected for recording or stimulation, and all exposed tissues were covered with warm mineral oil equilibrated with 95% oxygen and 5% carbon dioxide and retained by skin flaps in the usual way. Body temperature was maintained between 36 and 39° C by heating the experimental room and by the use of an infra-red lamp and an electrically-heated table. When 'natural' stimulation of cutaneous or muscle receptors was employed, denervation of most of the hind limb except for the chosen receptive fields was usually practised.

Responses of individual tract neurones were recorded by means of Ling–Gerard type glass capillary micropipettes filled with 3 M-KCl, with tip diameters of 0·5 μ or less. They were inserted by means of a rigidly mounted micromanipulator either into an otherwise undisturbed region of the dorsolateral tract usually in the 8th, 9th or 10th thoracic segment, or into more caudally situated regions of the grey matter containing the parent cell bodies of tract fibres. The usual cathode-follower input stage and standard dual-channel amplifying and display equipment were used, as described in other publications from this laboratory (Brock, Coombs & Eccles, 1952; Mark & Steiner, 1958; McIntyre, Bradley & Brock, 1959). Permanent photographic records were made with a Grass automatic camera. Electrical stimulation was effected either by thyratron-controlled condenser discharges and an isolating transformer, or by 'square-wave' pulses from a Grass stimulator and a radio-frequency stimulus isolation unit. For 'in-volume' stimulation of the spinal cord, pairs of fine steel needles, insulated except for the tips, were employed; otherwise, platinum or silver hook electrodes were used for stimulating or recording from peripheral nerves, spinal roots or the dissected dorsolateral tract. In some experiments contractions of hind-limb muscles were recorded by a strain gauge, the output of which drove one beam of the display unit by way of a direct-coupled amplifier. Muscle stretch was applied by a hand-operated rack and pinion device carrying the strain gauge, and the skin was stimulated by means of a blunt-ended glass rod or a fine probe.

'Input' was monitored either triphasically by a wick lead in contact with the appropriate dorsal root fibres at their entry into the spinal cord, together with an indifferent electrode placed on nearby muscle, or in the case of cutaneous nerves, diphasically by two electrodes located central to the stimulating pair (Mark & Steiner, 1958). Throughout this account, the terminology for cutaneous afferent fibres recently adopted by Lloyd (1957) will be used, namely, Group II for the band of larger myelinated fibres of about 6 μ and upwards in diameter (alpha and beta in the terminology of Gasser), and Group III for the smaller myelinated fibres or delta pile (Gasser, 1941).

RESULTS

Responses of the whole tract

Pattern and latency of responses. Figure 1 shows examples of the potentials evoked in the dorsolateral funiculus upon stimulating primary afferent fibres in lumbosacral dorsal roots. The most prominent feature is the large monosynaptically generated initial deflexion, which represents the nearly synchronous discharge of many tract fibres of rapid conduction velocity (Lloyd & McIntyre, 1950). The remainder of the response, or

after-discharge, is revealed most clearly by the monophasic method (*C* and *D*); it continues with declining amplitude for at least 10 msec.

In view of differences in conduction distance to the recording station, latency of the initial monosynaptic response varies, of course, from preparation to preparation; the range for maximal dorsal root volleys in these experiments was 1·7–2·8 msec. In a given preparation little variation in latency is observed when maximal volleys are used; however, when the

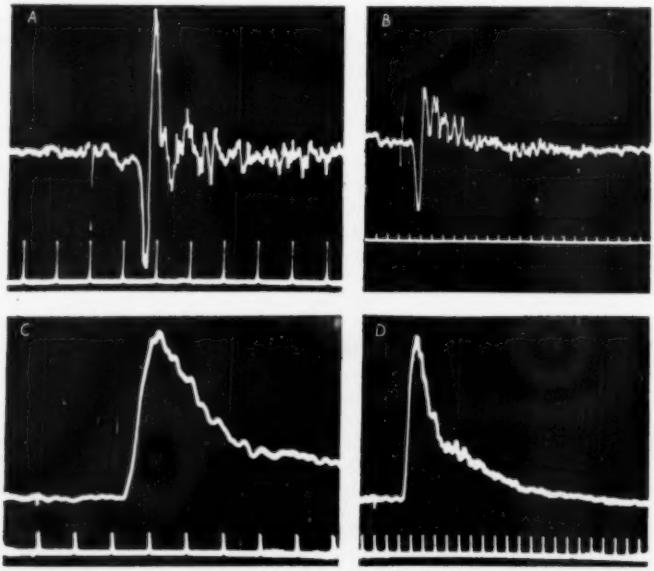


Fig. 1. Responses of dorsolateral tract to stimulation of combined L7 and S1 dorsal roots. *A* and *B* recorded by surface lead from the undisturbed tract in the 9th thoracic segment; two different preparations. *C* and *D*, monophasic records at different sweep speeds from the dissected dorsolateral funiculus in another preparation, at the 8th thoracic segmental level. *A* and *B* retouched. Time marker, msec.

input volley is reduced in size latency increases and becomes more variable. Graphical expression of this finding is presented in Fig. 2, in which the ordinate represents latency, and the abscissa, amplitude of the input dorsal root volley expressed as percentage of maximum.

Figure 3 gives examples of responses recorded from the whole dorsolateral funiculus upon stimulation of nerves in the hind limb. The response to a muscle afferent volley (*A*, *B*, *C*) is seen to consist of a well synchronized deflexion some 2–4 msec in duration, with very little after-discharge. Figure 3*D* demonstrates that a volley in a cutaneous nerve also sets up a substantial response in the dorso-lateral tract, in agreement with

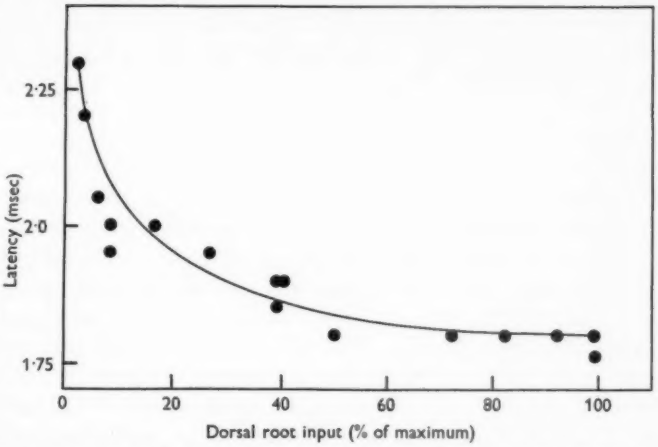


Fig. 2. Latency of tract response to stimulation of the combined L7 and S1 dorsal roots plotted against size of afferent volley. Ordinate, latency measured from the moment at which the afferent volley enters the cord. Abscissa, amplitude of dorsal root volley expressed as percentage of maximum.

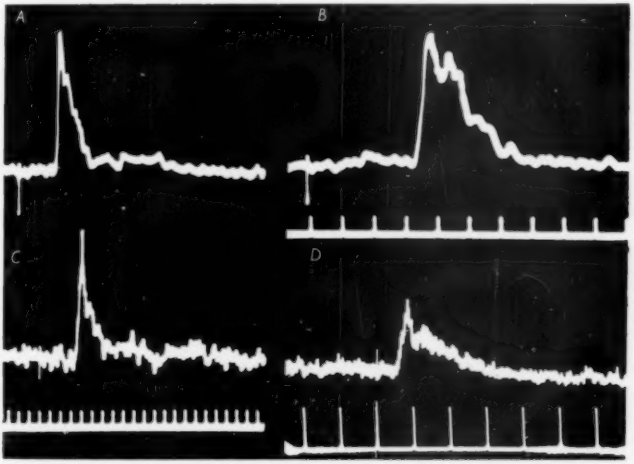


Fig. 3. Responses evoked by stimulation of hind-limb nerves and recorded monophasically from the ipsilateral dorsolateral funiculus. *A* and *B*, response recorded at two different sweep speeds to a volley in the combined nerves of triceps surae, plantaris and flexor digitorum longus, stimulus intensity being adequate to excite Group II as well as Group I fibres. *C*, response to similar stimulation of triceps surae nerves alone. *D*, response to stimulation (maximal for Group II fibres) of peroneal cutaneous nerve. Amplification for *C* and *D* twice that for *A* and *B*. Time scale below record *D* shows 10 msec intervals, and applies only to this record. Other time marks show milliseconds, and apply to the tracings above them. *A* and *C* recorded at the same sweep speed.

Laporte *et al.* (1956*a*). In comparison with the response to a muscle afferent volley, latency is longer, the initial deflexion is smaller and less well synchronized, and there is a prominent delayed discharge. It therefore appears that the after-discharge elicited in the tract by a dorsal root volley must largely be generated by the action of impulses in primary afferent fibres pertaining to cutaneous nerves, and not (or to a much smaller extent) by those in afferent fibres serving muscle. The tract response evoked by the combined action of a skin and a muscle-nerve volley timed to arrive simultaneously at the cord resembles the pattern of discharge evoked by dorsal root stimulation, and the combined potential approaches in magnitude and contour the sum of the tract responses to skin and muscle volleys in isolation. Thus excitatory convergence of cutaneous and muscle afferent fibres on to common tract units does not appear to be a prominent feature of the synaptic mechanisms concerned.

Although the latency of tract responses to cutaneous nerve stimulation is longer than that for a muscle afferent volley, measurement shows latency within the cord to be about the same, the additional delay in the case of cutaneous nerve volleys being the result of slower peripheral conduction velocity. Thus the earliest portion of the tract deflexion to be expected if cutaneous fibres could be stimulated in isolation at dorsal root level would appear early enough to contribute to the initial tract spike actually set up by a dorsal root volley, and it follows, too, that at least some of the cutaneous afferent fibres activating neurones projecting rostrally in the dorsolateral funiculus must do so by way of a single synaptic relay.

Upon repetitive stimulation of dorsal roots or hind-limb nerves, many tract neurones continue to respond even at fairly high frequencies of presynaptic drive. Surprisingly little reduction in the amplitude of individual tract responses takes place at a stimulation rate of 100/sec or even higher, in contrast with the monosynaptic discharge of motoneurones which is heavily depressed or abolished at such rates of presynaptic activation. However, the tract after-discharge suffers considerable reduction during a period of tetanic primary afferent stimulation.

Input–output relations. Input–output curves for the triphasically recorded monosynaptic initial tract deflexion in response to a dorsal root volley have previously been described (Lloyd & McIntyre, 1950). However, it is possible with monophasic recording to assess total tract output, including the after-discharge, by measuring area of the response. Figure 4 presents curves from an experiment in which measurements of discharge area (○) as well as amplitude of the initial spike-like deflexion (●) have been made from monophasic records of the tract discharge at various levels of dorsal root afferent input. Under the conditions of this experi-

ment, maximum input represents the summed activity of the Group I and Group II fibres in the stimulated dorsal roots (Lloyd, 1943*a*, *b*). Both curves rise steeply from their origin in expression of the fact that the smallest input volleys evoke measurable responses in the tract. From this it can be concluded, in confirmation of earlier studies, that dorsal root fibres of the lowest threshold excite some neurones of the tract by way of synaptic junctions of high transmitter potency, little or no summation being required to secure post-synaptic discharge. The curves stand in striking contrast with those defining amplitude of monosynaptic ventral

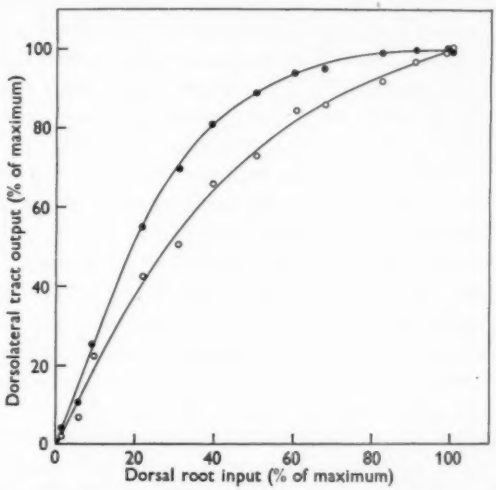


Fig. 4. Input–output curves relating amount of monophasically-recorded dorsolateral tract discharge to size of causative afferent volley, secured by stimulation of the combined L 7 and S 1 dorsal roots. Ordinates, tract output expressed as percentage of maximum; abscissa, amplitude of dorsal root volley also expressed as percentage of maximum. ●, amplitude of initial tract deflexion; ○, area of total tract discharge (potential-time integral). Each point the average of 8–12 observations.

root reflex discharge in response to various levels of dorsal root input, the sigmoid form of which expresses the need for summation in this simplest of spinal reflex paths (Lloyd, 1943*a*).

With monophasic recording we have found that amplitude of the initial spike tends to reach a plateau when the afferent volley attains about 70–80 % of maximum. Total tract discharge, however, continues to grow until the input reaches 100 % (Fig. 4, ○). Together these curves show that dorsal root fibres of all threshold values represented on the input scale (Groups I and II) effect excitatory connexions with neurones projecting by way of the dorsolateral funiculus, but that only the larger fibres of

lower threshold are concerned in evoking the early responses which sum to form the prominent initial component of the tract discharge. Of these larger fibres, those in the Group I category are clearly of prime importance. However, the larger Group II fibres of cutaneous origin must also participate in setting up the initial tract deflexion evoked by dorsal root stimulation, in view of their low threshold, and the fact that cutaneous afferent volleys can elicit tract responses of similar central latency (Fig. 3*D*).

The increase in total response of the tract (curve defined by open circles in Fig. 4) through recruitment of the higher-threshold Group II fibres to the input must obviously affect only its later portion. Indeed, progressive growth in the amount of late discharge can be observed by simple inspection of monophasic records as the input volley is increased above 50% and approaches its maximum value. No such growth is usually apparent in triphasic records made from the cord surface, an observation which suggested to Lloyd & McIntyre (1950) that the Group I band of fibres alone is responsible for the delayed as well as for the initial tract discharge. This difference may be a result or the tendency towards mutual cancellation of asynchronous unit potentials recorded in volume.

Figure 5 plots the input–output relation for the amplitude of the tract discharge evoked by muscle afferent volleys in four experiments, the abscissa showing size of Group I volley as percentage of maximum. It can be seen that the relation is essentially linear, some response of the tract being detectable with quite small Group I volleys, and that no plateau appears as is the case when a dorsal root provides the input channel. Increasing the stimulus strength to include afferent fibres of higher threshold does not augment amplitude of the evoked tract spike, but may add a small late component to the response. The curve in Fig. 5 thus provides confirmation of the conclusion from studies with dorsal root stimulation that fibres throughout the Group I range are effective in provoking discharge of tract neurones by way of synaptic junctions of high transmitting power. The curve contrasts with the relationship found when Group I input is plotted similarly against monosynaptic ventral root reflex discharge (Hunt, 1955), an example of which is shown by the interrupted line in Fig. 5.

The difference between the curve of Fig. 5 and the upper (amplitude) curve of Fig. 4 is presumably related in part to the absence, in the measured input for the former (Group I action only), of the Group II component which accounts for growth of a dorsal root volley from 50 to 100% of maximum, and which has little effect on the initial tract discharge. Since the Group I component of a dorsal root volley is compressed into the region of the abscissa between threshold and about 50% of maximum, a hypothetical curve defining growth of the fraction of tract response

caused by the Group I content of such a volley would obviously rise more steeply than is the case in Fig. 5. However, it is likely that discharge of tract units linked to the larger Group II fibres contributes towards bringing about the steeper ascent of curves showing growth of response to dorsal root volleys (Fig. 4), for the tract response to a cutaneous volley rises very abruptly with increase of the input above threshold (Fig. 6).

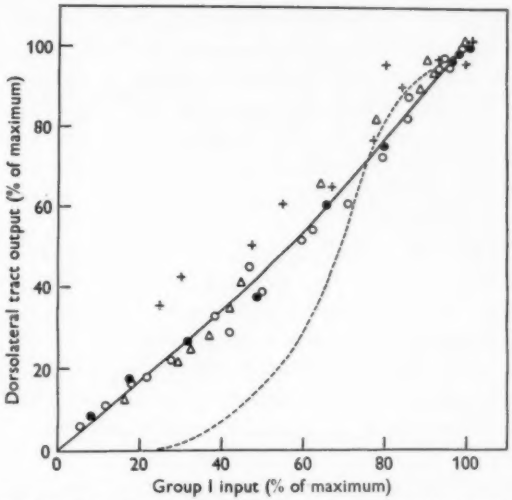


Fig. 5. Plotted points show for four experiments the amplitude of dorsolateral tract discharge (initial deflexion) expressed as percentage of maximum (ordinate) at various levels of Group I muscle afferent input, also expressed as percentage of maximum (abscissa). +, output measured triphasically from the undisturbed tract in response to input secured by stimulation together of the nerves to triceps surae, plantaris and flexor longus digitorum. ●, ○, two different preparations in which the same combination of muscle nerves provided the input, but output was recorded monophasically. △, another experiment with monophasic recording of output, but with input restricted to nerves supplying triceps surae. Each point the average of 10–20 observations. The interrupted curve shows the relationship obtained in another preparation between amplitude of Group I input and monosynaptic reflex discharge in the L 7 ventral root upon graded stimulation of the combined triceps surae, plantaris and flexor longus digitorum nerves (individual points not shown).

In one of the experiments plotted in Fig. 5, the Group I fibres stimulated were those of triceps surae alone (points shown by triangles), in contrast with the other three in which several muscle nerves were stimulated together. The points from the experiment with input restricted to a single synergic group do not appear to be distributed differently from those obtained by stimulation of the combined nerves of several muscles. This

observation, and the absence of a plateau in the curve, together suggest that there is not much convergent overlap of Group I afferent fibres from different muscles upon common tract neurones. Occlusion stemming from such convergence, if extensive, would be expected to find expression in a plateau, or at least an upward convexity, of the curve derived from the more massive input. Evidence from responses of single units in the tract favours the notion that on the whole individual muscles or synergic muscle groups are fairly discretely represented in the tract.

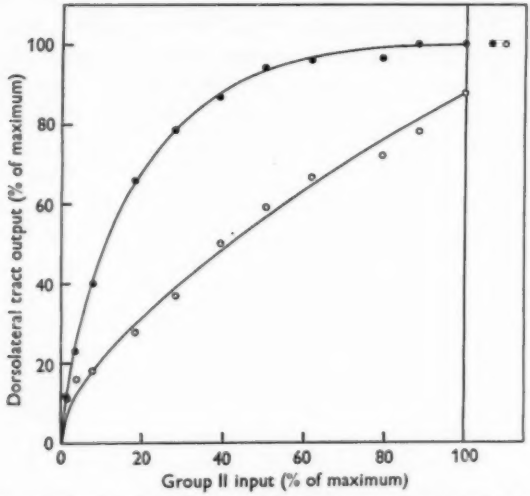


Fig. 6. Input–output curve for monophasically recorded tract discharge in response to graded stimulation of the ipsilateral superficial peroneal nerve. ●, amplitude of the initial deflexion; ○, area (potential-time integral) of whole tract discharge, both expressed as percentage of maximum (ordinate), against amplitude of afferent volley expressed as percentage of the maximal Group II (alpha-beta) spike. Points to the right of the vertical line at 100 % on the abscissa show the tract output evoked by stimulation strong enough to engage Group III (delta) fibres.

Figure 6 shows the input–output relation in an experiment with the peroneal cutaneous nerve serving as afferent channel, the tract response being recorded monophasically. The input scale represents amplitude of the cutaneous Group II volley in percentage of maximum; points to the right of the vertical line at 100 % show the effect of adding a substantial Group III component to the volley. As is the case with dorsal root stimulation, measurable responses of the tract appear with the smallest detectable cutaneous afferent volley, and increase steeply as the latter grow in size. The amplitude curve (●) reaches a plateau when the afferent volley is

about 70 % of maximum, and no further increase is observed when Group III impulses are added to the input. The curve for area, on the other hand, (○), shows after its steep initial portion a more gradual increase which continues throughout growth of the input volley to include all Group II fibres; recruitment of Group III fibres brings about a further increment in the total amount of discharge.

The abrupt origin of the curves in Fig. 6 shows that the low-threshold dorsal root afferent fibres effecting powerful synaptic linkages with tract neurones include many of cutaneous origin in addition to Group I fibres. The steeper rise and upward convexity of these curves compared with the roughly linear relationship obtained with Group I input (Fig. 5) suggests that the manner of synaptic linkage between cutaneous afferent fibres and neurones of the tract differs in some respects from the arrangement obtaining for the low-threshold muscle afferents. Shape of the amplitude curve in Fig. 6 is consistent with the notion that individual cutaneous afferent fibres make excitatory connexions with a considerable number of tract neurones, powerful enough with one or a few to cause discharge, subliminal in action on the remainder; and that the synaptic scale of each tract neurone in this category is composed of endings derived from many different primary afferent fibres. Otherwise expressed, both divergence and convergence are prominent features of the synaptic mechanisms linking cutaneous afferent fibres with tract neurones, in contrast with the more discrete arrangement postulated for Group I muscle afferents. Such an arrangement could explain the 'amplification' indicated by the steeply rising portion of the upper curve in Fig. 6, for each additional afferent fibre recruited by progressively stronger stimulation would be able to fire more neurones than it could acting in isolation, by virtue of spatial summation in units of the subliminal fringe created by action of its fellow afferent fibres of lower threshold. The plateau is likely to be at least in part a result of occlusion. The existence of extensive functional convergence upon individual tract neurones is directly demonstrated by the unit responses to be described subsequently.

Post-tetanic potentiation. Figure 7 demonstrates that there is a striking difference between the degree of post-tetanic potentiation (PTP) detectable in a motoneurone pool (Lloyd, 1949) and a population of dorsolateral tract neurones. Above (○) is plotted the amplitude of a monosynaptic reflex discharge recorded from the L7 ventral root and elicited at four-second intervals by test stimulation (supramaximal for Group I fibres) of a group of muscle nerves. Below is plotted (●) the amplitude of the initial dorsolateral tract deflexion evoked by the same afferent volley and recorded simultaneously on the second oscilloscope beam. The tract response undergoes very little potentiation (peak 1·5 times control value)

in comparison with the motoneurone discharge which is increased twenty-fivefold. This result agrees with the findings of Holmqvist, Lundberg & Oscarsson (1956), and shows that there is a small subliminal fringe of tract neurones even with a maximal Group I volley, in contradiction of earlier experiments using the less sensitive 'in-volume' recording technique, in which no PTP could be detected (McIntyre, 1953). Figure 7

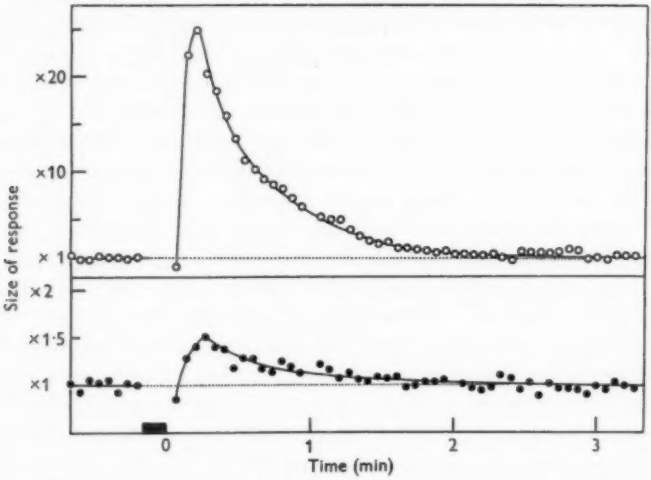


Fig. 7. Post-tetanic potentiation of responses to stimulation (supramaximal for Group I fibres) of the combined triceps surae, plantaris and flexor longus digitorum nerves. Ordinates, size of individual responses in multiples of pre-tetanic control amplitude (note different scales). Abscissa, time in minutes after cessation of tetanic stimulation for 10 sec at 450/sec, delivery of which is indicated by the black rectangle. Individual controls shown to the left of the latter. ○, monosynaptic reflex discharge in L7 ventral root. ●, amplitude of monophasically recorded dorsolateral tract response recorded simultaneously on the second oscilloscope beam.

demonstrates that the two sets of Group I presynaptic endings undergo qualitatively similar post-tetanic changes in transmitter potency, but the large discrepancy in magnitude of the effect emphasizes the difference between the two synaptic systems. Even less PTP has been seen in responses of the tract to maximal cutaneous afferent volleys, indicating that the subliminal fringe for the appropriate tract neurones must be very small indeed under these test circumstances.

Responses of single tract units

Response patterns. With Ling–Gerard micropipettes, responses can be recorded from single fibres in the dorsolateral funiculus, from cells of

Clarke's column in the upper lumbar segments, or more caudally from other cells in the grey matter, the units being identified in each case by their short-latency antidromic response to stimulation of the dorsolateral, funiculus in the mid or upper thoracic region. Most of our unit responses have been recorded from tract fibres, as in the work of Laporte *et al.* (1956*b*), with whose results our own are in general agreement. Figure 8 shows typical examples of single-tract unit responses to dorsal-root stimulation. The most striking characteristic of the responses to single afferent volleys is the common occurrence of repetitive firing, in contrast

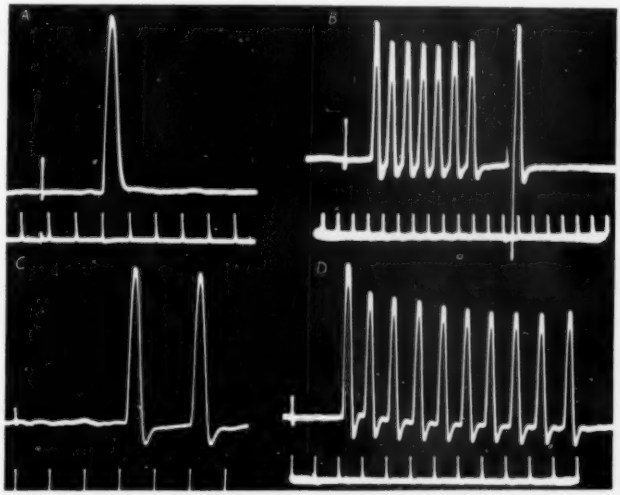


Fig. 8. Responses recorded by Ling–Gerard micropipettes of four different fibres in the dorsolateral funiculus to stimulation (maximal for Group I and Group II fibres) of the combined L7 and S1 dorsal roots. The last spike in record *B* is set up by direct stimulation of the tract several segments above the recording site; its short latency shows it to be an antidromically conducted response. Spikes retouched in *A*, *C* and *D*. Time marker, msec.

with the behaviour of motoneurones. Firing frequency was often as high as 800/sec and in many units initially exceeded 1000/sec. Such high frequencies might suggest injury, but this can be ruled out because no high-frequency bursts appeared spontaneously, and such neurones could be made to discharge single impulses by reducing the size of afferent volley (Figs. 10 and 11) or by antidromic activation (last spike in Fig. 8*B*). Furthermore, direct stimulation of such tract neurones by 'square-wave' current pulses 10–50 msec in duration induced repetitive firing throughout the stimulus, initially at high frequency, whereas a brief shock evoked but one impulse.

Thus there can be no reasonable doubt that the repetitive impulse trains are physiological at least in the sense that they can be evoked by way of essentially normal pre- and post-synaptic elements, and that they reflect some property of the synaptic junctions concerned which is absent from, or fails to manifest itself at, the synaptic junctions engaging motoneurones. Acceptance of these firing patterns as a normal manifestation of tract unit activity is further suggested by the prolonged shower of potentials recordable from the whole dorsolateral funiculus (Fig. 1), a ready explanation for which would be the occurrence of repetitive action in the undisturbed projection system.

By summing a series of unit discharge patterns, an approximate synthesis can be made of the total tract response which can be compared with an actual monophasic tract discharge. Figure 9 presents such a comparison carried out with the aid of certain simplifying assumptions explained in the legend. Peaks of the rectangles indicate contour of the summed response of 77 tract units; superimposed on this is a tracing of an actual whole-tract discharge recorded monophasically. Despite the relative crudity of the synthesis, correspondence between the actual monophasic response and the hypothetical discharge is sufficiently close to suggest not only that this small sample of 77 units is reasonably representative of the population, but also that repetitive firing of individual neurones is indeed an adequate explanation for the tract after-discharge.

Effects of grading volley size on unit responses. Figure 10 presents four responses of a tract neurone to a dorsal root volley of increasing amplitude, and demonstrates our regular finding with repetitively-firing units of reduction in latency and increase in number of discharges with increased presynaptic drive. Figure 11 shows graphically for another unit similar changes in latency (●) and spike number (▲), and it can be seen that these two effects are to some extent independent. Reduction in latency with augmentation of input is also seen in those units yielding only one impulse to a maximal afferent volley. It is obvious that these effects of grading the amplitude of afferent input on latency and on the number of post-synaptic impulses together demonstrate convergence of action by impulses in afferent fibres of different threshold upon individual tract neurones.

From experiments in which nerves in the hind limb rather than dorsal roots were stimulated it is clear that the majority of tract units responding to dorsal root stimulation with more than 2–3 impulses, and all those discharging long trains (e.g. Fig. 8*B*, *D*), are connected to afferent fibres of cutaneous nerves, whereas a large proportion of those with responses limited to 1 or 2 impulses are concerned with signals from muscle stretch receptors. This is in general agreement with the findings of Laporte *et al.* (1956*b*), and with the results of experiments upon the whole-tract dis-

charge (Fig. 3). Our experiments indicate that different muscles or synergic groups are fairly discretely represented in the tract, whereas widespread convergence takes place on units linked to cutaneous afferent fibres.

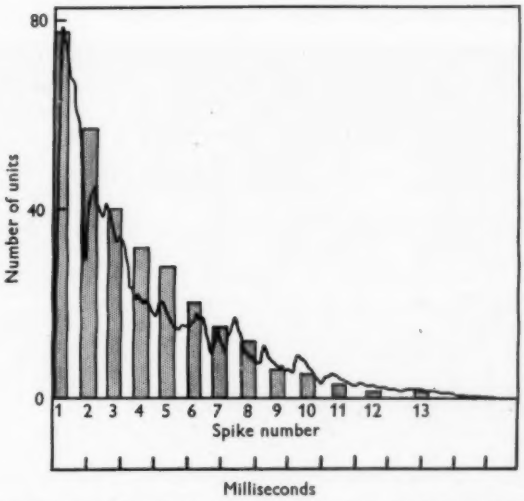


Fig. 9. Comparison of 'synthetic tract response', derived from firing patterns of 77 units, with actual monophasic dorsolateral tract discharge in response to maximal stimulation of L7 and S1 dorsal roots. Ordinate, number of units contributing to size of supposed summed potential at each of 13 intervals after onset of the initial spike, the intervals being set by the temporal sequence of the 13 impulses discharged by the unit giving the longest repetitive train (upper abscissa scale). Time (msec) after onset of first spike or whole tract discharge shown below. Stippled rectangles located on abscissa in accordance with the sequence of spikes in the unit firing 13 times; height of each is determined by the number of impulses appearing in that position were all 77 units to fire simultaneously with the same initial latency, and with the temporal sequence of subsequent spikes, if any, corresponding to that of the unit giving the longest train. Continuous line, tracing of an actual monophasic tract response to dorsal root stimulation on same time base and scaled so that its peak corresponds to height of the first rectangle. Impulses from different units are assumed to contribute equally to recorded potential.

Unit responses to receptor stimulation. Further subdivision of the tract neurones linked with Group I afferent fibres is indicated by their responses to stretch of muscles with intact innervation. In agreement with Laporte & Lundberg (1956), the majority exhibited spindle behaviour but some had the characteristics of tendon organs. Low mechanical threshold and 'in-parallel' behaviour during active muscle contraction (Matthews, 1933) together with, in the few instances in which it was tried, acceleration of

discharge by the action of succinylcholine or decamethonium (Granit, Skoglund & Thesleff, 1953) served to identify spindle-controlled tract units (Fig. 12*A*); the tendon-organ type of unit was so designated because of high mechanical threshold and 'in-series' behaviour (Fig. 12*B*). In our experiments no evidence was obtained suggesting convergence of the two

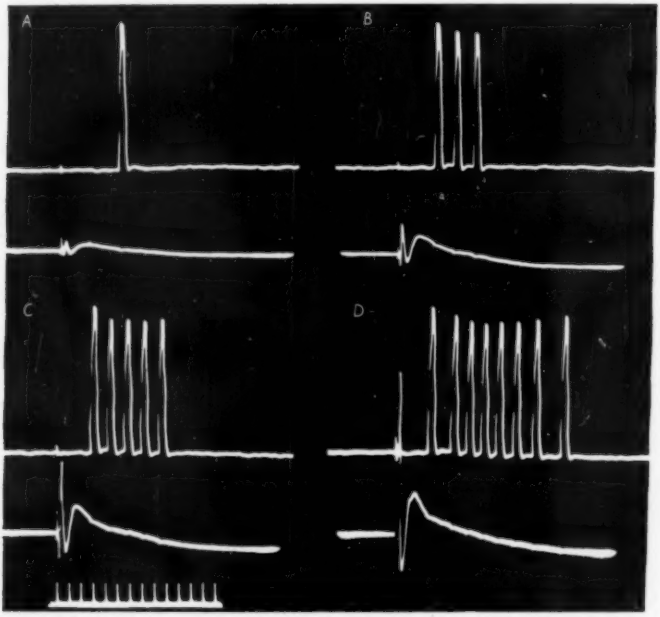


Fig. 10. Responses of a single unit in the dorsolateral funiculus to four different levels of afferent input secured by stimulation of the ipsilateral L7 dorsal root. Upper tracings, unit discharge recorded by Ling–Gerard micropipette; lower records, afferent volley recorded by surface lead at its entry into the cord. *D*, volley maximal for Groups I and II; *A*, *B*, *C*, volley 4·5%, 17% and 42% of maximum respectively; spikes retouched. Time marker, msec.

types of Group I fibre upon common tract neurones (but cf. Lundberg & Oscarsson, 1956). We conclude that the dorsolateral tract not only provides for discrete representation of synergic muscle groups, but within each group separate lines of communication are available for impulses from the two types of stretch receptor.

Tract units fired by electrical stimulation of skin nerves (Fig. 13*A* respond also to low-intensity mechanical stimulation of the integument or its appendages (Fig. 13*B*). The receptive fields serving such units have varied greatly in extent, some measuring many hundreds of square millimetres, whereas others, especially on the foot or toes, were only a few

millimetres across. Bending of hairs, or pressure exerted on the skin, were effective in setting up discharge of tract units linked to the area in question. The initial burst of impulses in response to natural stimulation may be at very high frequency, as is shown in Fig. 13*B*, *C*. Thus it appears that the rapid repetitive firing set up by electrically-initiated nerve volleys is not entirely unnatural, almost as high rates of firing being evoked by receptor-initiated activity, presumably in converging primary afferent fibres serving the same skin area.

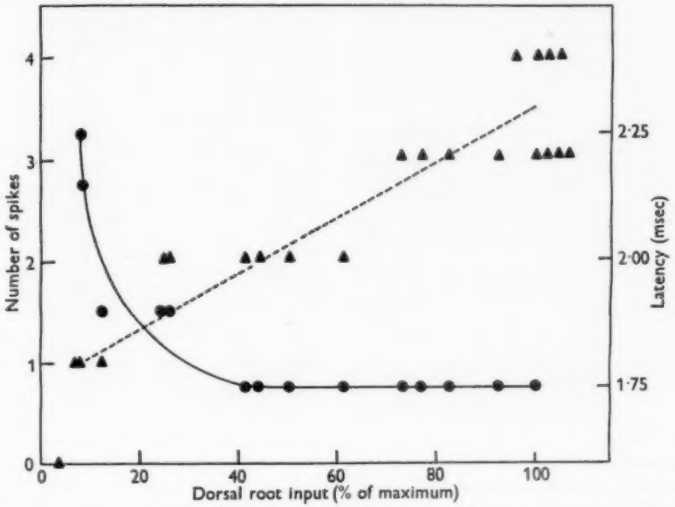


Fig. 11. Latency of first spike msec; (● and continuous line, right-hand ordinate scale), and number of spikes appearing (▲ and interrupted line, left-hand ordinate scale) are plotted for a dorsolateral tract unit at various levels of afferent input evoked by stimulation of the L 7 and S 1 dorsal roots and expressed as percentage of maximum (abscissa).

Inhibition. In addition to the powerful excitatory effects of afferent impulses on neurones of the dorsolateral tract, inhibitory action can also be observed, as described by Laporte *et al.* (1956*b*) and Laporte & Lundberg (1956). Inhibition of tract units with excitatory drive from a given muscle may be exerted by Group I impulses from its antagonist, or by action in afferent fibres of higher threshold in other deep or superficial nerves. Although impulses from different cutaneous nerves converging upon a tract neurone commonly exert excitatory effects, inhibitory interaction may also be observed. The experiment with natural stimulation illustrated in Fig. 13*D* may indicate the significance of such inhibition. This unit, fired by stimulation of the superficial peroneal nerve, showed

some background discharge and responded to pressure on the lateral toe (upper record). It was readily inhibited by light pressure on the neighbouring toe or the plantar cushion (lower record). Such inhibition appears to be of the kind reported for other afferent pathways such as the dorsal column system (Mountcastle & Powell, 1959), and could serve to sharpen spatial discriminative ability.

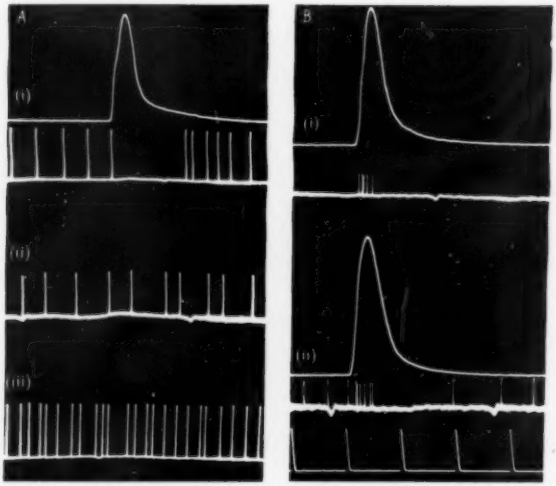


Fig. 12. Responses of tract units linked to stretch receptors in triceps surae, the afferent innervation of which was intact. *A*, unit showing muscle spindle behaviour: (i) lower record shows unit discharging to slight stretch of the muscle (about 15 g) and pausing during contraction brought about by a single weak stimulus to the peripheral end of the severed S 1 ventral root—upper trace is strain gauge record of tension developed at the tendo Achillis; (ii) resting discharge of the same unit with similar slight tension on the tendon; (iii) the same unit a few minutes after intravenous injection of 200 μg of decamethonium bromide, muscle length unchanged; alterations in spike size due to slight movement of micro-electrode tip. *B*, records similar to those of *A* (i), from another unit showing tendon organ characteristics: (i) at moderate initial tension; (ii) after increasing tension on the tendon to the point of evoking discharge of the unit. Time marker, 100 msec.

DISCUSSION

The origin, destination and physiological role of all the elements contributing to the responses recorded from the dorsolateral funiculus at present remains speculative. However, there can be little doubt that at least a major fraction of the neurones responding to impulses of muscle receptor origin belong to the classical dorsal spinocerebellar or dorsolateral tract of Flechsig, with cell bodies in the column of Clarke and end-stations in the cerebellum. Indeed, unit responses in the region of Clarke's column

to antidromic and orthodromic stimulation have been recorded in a few experiments of the present series, and recently by Curtis, Eccles & Lundberg (1958), which confirm this view, though the great susceptibility to injury of the cells in this nucleus makes systematic study very difficult. The significance of the neurones connected to afferent fibres of cutaneous

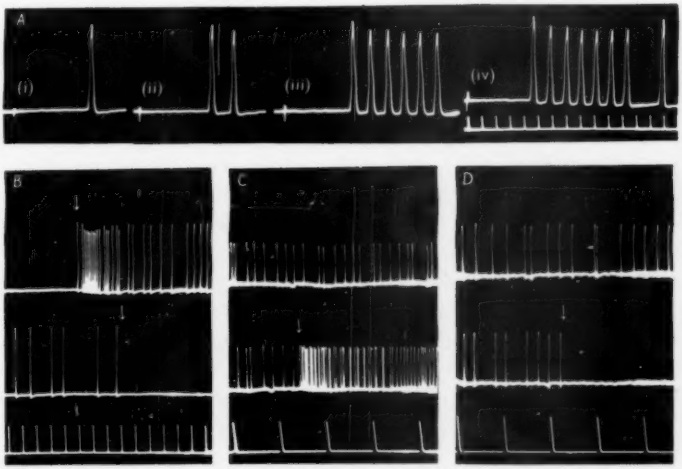


Fig. 13. Responses of units in the dorsolateral funiculus to cutaneous nerve or skin receptor stimulation. *A* (i)–(iv), a series of responses of a unit to stimuli of increasing strength applied to the intact peroneal cutaneous nerve; stimulus in (iv) strong enough to engage some Group III fibres. Time marker, msec. *B*, discharge of the same unit to light touch in the centre of its receptive field (skin in front of ankle joint); arrow in upper record signals onset of stimulation; in lower record, arrow shows cessation of stimulus some seconds later. Time marker, 10 msec. *C*, another unit, showing spontaneous discharge in the absence of stimulation (upper record); arrow in lower record shows onset of tactile stimulus to receptive field as in *B*. Time marker, 100 msec. *D*, another unit showing some spontaneous discharge, and responding to pressure on the lateral surface of the 4th digit with a well-maintained discharge (upper record); arrow in the lower record signals the onset of tactile stimulation of the plantar cushion, which inhibited the unit's firing. Time marker, 100 msec.

origin is, however, not yet apparent. The large measure of independence of tract neurones fired by muscle and cutaneous receptors respectively suggests that each belongs to a separate functional system, and this view is reinforced by our failure to record from the region of Clarke's column any of the long repetitive trains of impulses characteristic of skin-controlled units, even with maximal stimulation of dorsal roots. However, such firing patterns are encountered in the grey matter of segments between the level of afferent volley entry and the caudal end of Clarke's column. Thus

there is good reason to suppose that many of the tract neurones linked to cutaneous afferent fibres do not belong to the dorsal spinocerebellar system. Whether they pertain to the spino-olivary tract described by Grundfest & Carter (1954), the fast lateral-column system with eventual projection to the cerebral cortex (Morin, 1955; Mark & Steiner, 1958), or are simply long propriospinal neurones cannot at present be decided with certainty. Lundberg & Oscarsson (1959) have recently reported that some dorsolateral tract fibres linked to cutaneous afferents have their cell bodies below the level of Clarke's column and do not project to the cerebellum, but according to these authors other skin-controlled fibres do belong to the dorsal spinocerebellar system. At any rate, there appears to be agreement that fibres with a diversity of function course rostrally in the anatomical field of Flechsig's tract.

Despite the probable functional non-homogeneity of the various neurones contributing to the responses we have studied in the dorsolateral funiculus, certain similarities in their behaviour to synaptic activation stand out in comparison with the more extensively studied synaptic responses of motoneurones. Outstanding amongst the properties common to these afferent synapses is the small amount of summation required to discharge the post-synaptic neurones as compared with motoneurones, as shown by the input–output relationship at low levels of input. Another difference (doubtless related to the last) between the synapses of motoneurones and those of the tract neurones is the much smaller subliminal fringe detectable in the latter with maximal presynaptic volleys, a difference revealed by comparing the curves of post-tetanic potentiation (Fig. 7). The remarkable resistance of the afferent synapses to anaesthetic agents may be cited as a third characteristic contrasting with the much greater susceptibility of motoneurone synapses, and the capacity of post-synaptic tract neurones to continue responding during relatively high-frequency repetitive presynaptic stimulation constitutes a fourth distinctive property not apparent in the responses of motoneurones.

Although the various tract neurones we have studied are linked with primary afferent fibres by synapses which are similarly powerful according to the above criteria, nevertheless some differences appear to exist between the junctions made by afferent fibres of muscular and those of cutaneous origin. The apparently steeper rise and plateau of input–output curves for cutaneous nerve volleys as compared with the approximately linear relation for muscle afferent input suggests an even closer and more powerful linkage of the former with the appropriate tract neurones. Though other factors are doubtless involved in the genesis of this difference, a similar conclusion is indicated by the very small amount of post-tetanic potentiation detectable in the tract response to a maximal cutaneous nerve volley,

and by the prominence of repetitive discharge in the post-synaptic unit responses engendered by such afferent input. It appears likely that convergence of cutaneous afferent fibres upon common post-synaptic neurones is much more extensive than in the case of muscle afferent fibres, at least of those in the Group I range. An outstanding impression of our experience with tract unit responses elicited by stimulation of muscle nerves or stretch receptors is the infrequency with which such units can be fired from muscle afferents outside those belonging to a synergic pair or group. It seems likely that such muscles or synergic groups on the whole enjoy a fairly discrete representation in the tract, so that the projection system can convey to higher levels topographically organized information concerning the moment-to-moment patterns of posture or movement in the limb. In view of the remarkable giant synaptic contacts described by Szentágothai & Albert (1955) between primary afferent fibres and Clarke's column cells, which presumably are the actual junctions yielding the tract discharges evoked by Group I afferent input, it would be of great interest to know the morphological characteristics of the junctions between cutaneous afferent fibres and the tract neurones which they activate so powerfully.

Mechanism of repetitive firing. It is of interest to examine possible ways in which the rapid repetitive discharge of dorsolateral tract neurones may be brought about. It has already been pointed out that these repetitively firing cells have in all cases responsed with a single impulse when activated antidromically (Fig. 8*B*), or by just supra-threshold synaptic drive (Fig. 10*A*). These findings not only help to exclude injury as a factor in the genesis of multiple firing, but demonstrate conclusively that the mechanism responsible is not inherent in the post-synaptic cells themselves nor dependent upon self re-excitation by neurone chains driven by recurrent collaterals (Bishop, Jeremy & McLeod, 1953). In short, the observations demonstrate that the mechanisms responsible for repetitive firing are presynaptically located. Similar conclusions have been reached for repetitively-firing spinal internuncials by Hunt & Kuno (1959) and by Wall (1959). However, it is not easy to decide just what presynaptic machinery is responsible, for several factors could operate to prolong synaptic action. Simple dispersion of presynaptic impulses through differences in conduction velocity could lead to a relatively prolonged bombardment of second-order neurones, even with single-shock stimulation, and this almost certainly contributes to prolongation of action, especially with a volley set up in Group II or III fibres at some distance from the cord. However, with stimulation of dorsal roots near the cord this dispersion would be very much less, yet repetitive firing is very striking with afferent volleys set up in this way. Furthermore, we have

found that repetitive firing can be evoked by stimulation of primary afferent fibres in the dorsal column, and it does not seem possible to find a locus for such stimulation at which the repetitive firing is effectively reduced, though for a given tract unit such stimulation should, when near the site of synaptic relay, achieve minimal presynaptic dispersion. Thus, while admitting that simple dispersion may play a part in prolonging presynaptic action, especially when the afferent volley is set up in nerves of the limb, it does not seem possible to explain it fully in this way. The 'dorsal column relay' of Hursh (1940) is another possible mechanism which could prolong presynaptic action at these afferent synapses; however, with the spinal cord at 38–39° C Hursh's phenomenon is greatly reduced, yet repetitive firing is just as prominent as it is at lower cord temperatures.

The most likely possibilities are either repetitive presynaptic bombardment through delay-paths provided by presynaptic systems of internuncial chains (Lorente de Nó, 1938) or prolonged transmitter action by impulses in individual presynaptic terminals. The latter hypothesis could involve a prolongation of electrical action in the ultimate presynaptic ramifications (Lloyd & McIntyre, 1949), with consequent maintenance of transmitter action for considerably longer than the duration of an impulse in the parent axon, presumably by sustained delivery of a chemical agent; or the enduring transmitter action could be the result of glial or other juxta-synaptic barriers maintaining effective concentrations of chemical transmitter agent after its initial release over a relatively short time course. Evidence to hand does not permit proof or refutation of either of these suggestions, nor of the occurrence of repetitive bombardment via internuncial chains. However, the similar and very striking repetitive firing of Renshaw cells (Renshaw, 1946; Eccles, Fatt & Koketsu, 1954; McIntyre *et al.* 1956) in response to single antidromic volleys in motoneurones, a situation in which it seems unlikely that impulse-circulation in internuncial chains could play a part, inclines us to favour the view that prolonged transmitter action is an important part of the mechanism leading to multiple firing at afferent synapses. This hypothesis, and the postulate of repetitive bombardment through internuncial action, are by no means mutually exclusive; indeed, there is good reason to suppose that the latter mechanism, together with dispersion of primary afferent impulses (especially when conduction distance is long) also play a part in prolonging the synaptic drive. Hunt & Kuno (1959), for example, present evidence favouring the occurrence of a prolonged barrage of impulses upon repetitively-firing internuncials, which in many respects behave like the afferent neurones studied in this investigation. It seems likely to us that the later spikes of our repetitive trains may depend largely upon the

delayed arrival of presynaptic impulses, but that the initial burst of high-frequency post-synaptic discharge is more probably brought about mainly by prolonged intense transmitter action initiated by relatively synchronous impulses in many converging afferent terminals. Interjection of a directly-evoked (Wall, 1959; Hunt & Kuno, 1959), or an antidromic impulse into a synaptically-elicited train does not lead to 'resetting' of the rhythm, and this has been cited as evidence supporting the internuncial chain hypothesis (Wall, 1959). However, in the case of the repetitive trains studied by us, failure of an antidromic impulse to upset the spike rhythm only demonstrates the presynaptic locus, but not the nature, of the driving mechanism. Antidromic stimulation of our tract neurones, such as the one giving the responses of Fig. 8*B*, does not reset the rhythm, but firing rate is so high that the temporal sequence of impulses in the early part of the train is determined solely by recovery from refractoriness.

It is of interest that we have been able to elicit similar trains of impulses in tract neurones by direct stimulation with square-wave current pulses; in other words, these neurones show very little accommodation. The observation is consistent with the view that prolonged depolarization of post-synaptic cells by the sustained transmitter action postulated above could be the immediate agent for generating repetitive discharge, much in the manner shown for crustacean stretch receptor neurones by Eyzaguirre & Kuffler (1955). Apart from the presynaptic mechanisms, the ability to discharge at high frequencies shown by central neurones such as the one yielding the responses in Fig. 13*A*, *B*, or the internuncials studied by Haapanen, Kolmodin & Skoglund (1958), Wall (1959) and Hunt & Kuno (1959), provides another striking contrast with motoneurones, which seldom if ever respond in this way. The difference is probably due to the presence of a prolonged phase of subnormality in the latter which greatly limits their capacity to fire repetitively at high rates. This post-activation subnormality is associated with a well developed after-hyperpolarization, which is absent from internuncials (Hunt & Kuno, 1959), and probably from fusimotor neurones (Hunt & Paintal, 1958) which latter also tend to fire repetitively. One interpretation of the difference could be that repetitively-firing neurones do not exhibit post-activation hyperpolarization and associated subnormality because the impulses, initiated in or near the initial portion of the axon, do not invade the dendritic processes, the most likely site of the prolonged recovery processes in motoneurones which are so invaded (Lloyd, 1951).

The physiological significance of repetitive firing by neurones of the dorsolateral funiculus remains at present a matter for speculation. However, its common occurrence in second or third-order neurones of other afferent systems (e.g. Amassian, 1953; Bishop *et al.* 1953; Rose &

Mountcastle, 1954), and in spinal internuncials, cortical and reticular neurones (Amassian, 1953; Amassian & de Vito, 1954; Hunt & Kuno, 1959) suggests that the phenomenon may be of considerable importance in central nervous function, for example, through temporal expansion and hence intensification of post-synaptic action, or by synaptic transformation of spatial into temporal patterns of signals. The occurrence of repetitive action at so many central synapses, and the contrasting behaviour of motoneurones, suggest that the latter may represent an exceptional case, in keeping with the unique function of final-common-pathway cells.

SUMMARY

1. Responses of neurones projecting rostrally in the field of Flechsig's tract have been set up by stimulating primary afferent fibres or receptors pertaining to the ipsilateral hind limb, and recorded singly or *en masse*.

2. In the whole-tract response to dorsal root stimulation the initial spike-like component is monosynaptically evoked by impulses in Group I and low-threshold Group II fibres, and the after-discharge results largely from action in the smaller myelinated afferent fibres, especially those of cutaneous nerves.

3. Input–output curves demonstrate that the synaptic linkages between tract neurones and afferent fibres both of skin and muscle origin are powerful, little or no summation being required to secure post-synaptic discharge. Little post-tetanic potentiation is evident at these junctions, and transmission is well maintained during repetitive presynaptic stimulation.

4. Unit recording shows that a large proportion of tract neurones fire more than once in response to a single afferent volley, those fired by cutaneous impulses yielding the longest repetitive trains. Correspondence between an actual whole-tract response and a synthetic tract discharge constructed from unit firing patterns indicates that this repetitive firing accounts for the after-discharge.

5. Most of the tract neurones responding to cutaneous nerve volleys or stimulation of skin receptors are distinct from those fired by muscle afferent volleys; of the latter units, most are linked with muscle spindles but some with tendon organs. Synergic muscle groups appear to be fairly discretely represented in the tract.

6. In addition to the powerful excitatory synaptic effects, inhibitory actions also take place at the junctional mechanisms serving neurones of the dorsolateral tract.

7. The mechanism and significance of the repetitive firing phenomenon are discussed.

Generous assistance from the Medical Research Council of New Zealand is gratefully acknowledged.

REFERENCES

AMASSIAN, V. E. (1953). Evoked single cortical unit activity in the somatic sensory area. *Electroenceph. clin. Neurophysiol.* **5**, 415–438.

AMASSIAN, V. E. & DE VITO, R. V. (1954). Unit activity in reticular formation and nearby structures. *J. Neurophysiol.* **17**, 575–603.

BISHOP, P. O. (1953). Synaptic transmission. An analysis of the electrical activity of the lateral geniculate nucleus in the cat after optic nerve stimulation. *Proc. Roy. Soc.* B, **141**, 362–392.

BISHOP, P. O., JEREMY, D. & MCLEOD, J. G. (1953). Phenomenon of repetitive firing in lateral geniculate of cat. *J. Neurophysiol.* **16**, 437–447.

BROCK, L. G., COOMBS, J. S. & ECCLES, J. C. (1952). The recording of potentials from motoneurones with an intracellular electrode. *J. Physiol.* **117**, 431–460.

CURTIS, D. R., ECCLES, J. C. & LUNDBERG, A. (1958). Intracellular recording from cells in Clarke's column. *Acta physiol. scand.* **43**, 303–314.

ECCLES, J. C., FATT, P. & KOKETSU, K. (1954). Cholinergic and inhibitory synapses in a pathway from motor-axon collaterals to motoneurones. *J. Physiol.* **126**, 524–562.

EYZAGUIRRE, C. & KUFFLER, S. W. (1955). Processes of excitation in the dendrites and in the soma of single isolated sensory nerve cells of the lobster and crayfish. *J. gen. Physiol.* **39**, 87–119.

GASSER, H. S. (1941). The classification of nerve fibres. *Ohio J. Sci.* **41**, 145–159.

GRANIT, R., SKOGLUND, S. THESLEFF, S. (1953). Activation of muscle spindles by succinylcholine and decamethonium. The effects of curare. *Acta physiol. scand.* **28**, 134–151.

GRUNDFEST, H. & CAMPBELL, B. (1942). Origin, conduction and termination of impulses in dorsal spino-cerebellar tracts of cats. *J. Neurophysiol.* **5**, 275–294.

GRUNDFEST, H. & CARTER, W. B. (1954). Afferent relations of inferior olivary nucleus. 1. Electrophysiological demonstration of dorsal spino-olivary tract in cat. *J. Neurophysiol.* **17**, 72–91.

HAAPANEN, L., KOLMODIN, G. M. & SKOGLUND, C. R. (1958). Membrane and action potentials of spinal interneurones in the cat. *Acta physiol. scand.* **43**, 315–348.

HOLMQVIST, B., LUNDBERG, A. & OSCARSSON, O. (1956). Functional organisation of the dorsal spino-cerebellar tract in the cat. V. Further experiments on convergence of excitatory and inhibitory actions. *Acta physiol. scand.* **38**, 76–90.

HUNT, C. C. (1955). Monosynaptic reflex response of spinal motoneurones to graded afferent stimulation. *J. gen. Physiol.* **38**, 813–852.

HUNT, C. C. & KUNO, M. (1959). Properties of spinal interneurones. *J. Physiol.* **147**, 341–363.

HUNT, C. C. & PAINTAL, A. S. (1958). Spinal reflex regulation of fusimotor neurones. *J. Physiol.* **143**, 195–212.

HURSH, J. B. (1940). Relayed impulses in ascending branches of the dorsal root fibres. *J. Neurophysiol.* **3**, 166–174.

LAPORTE, Y. & LUNDBERG, A. (1956). Functional organisation of the dorsal spino-cerebellar tract in the cat. III. Single fibre recording in Flechsig's fasciculus on adequate stimulation of primary afferent neurones. *Acta physiol. scand.* **36**, 204–218.

LAPORTE, Y., LUNDBERG, A. & OSCARSSON, O. (1956*a*). Functional organisation of the dorsal spino-cerebellar tract in the cat. I. Recording of mass discharge in dissected Flechsig's fasciculus. *Acta physiol. scand.* **36**, 175–187.

LAPORTE, Y., LUNDBERG, A. & OSCARSSON, O. (1956*b*). Functional organisation of the dorsal spino-cerebellar tract in the cat. II. Single fibre recording in Flechsig's fasciculus on electrical stimulation of various peripheral nerves. *Acta physiol. scand.* **36**, 188–203.

LLOYD, D. P. C. (1943*a*). Reflex action in relation to pattern and peripheral source of afferent stimulation. *J. Neurophysiol.* **6**, 111–120.

LLOYD, D. P. C. (1943*b*). Neuron patterns controlling transmission of ipsilateral hind limb reflexes in cat. *J. Neurophysiol.* **6**, 293–315.

LLOYD, D. P. C. (1949). Post-tetanic potentiation of response in monosynaptic reflex pathways of the spinal cord. *J. gen. Physiol.* **33**, 147–170.

LLOYD, D. P. C. (1951). Electrical signs of impulse conduction in spinal motoneurones. *J. gen. Physiol.* **35**, 255–288.

LLOYD, D. P. C. (1957). Input–output relation in a flexor reflex. *J. gen. Physiol.* **41**, 297–306.

LLOYD, D. P. C. & MCINTYRE, A. K. (1949). On the origin of dorsal root potentials. *J. gen. Physiol.* **32**, 409–443.

LLOYD, D. P. C. & MCINTYRE, A. K. (1950). Dorsal column conduction of Group I muscle afferent impulses and their relay through Clarke's column. *J. Neurophysiol.* **13**, 39–54.

LORENTE DE NÓ, R. (1938). Analysis of the activity of the chains of internuncial neurones. *J. Neurophysiol.* **1**, 207–244.

LUNDBERG, A. & OSCARSSON, O. (1956). Functional organisation of the dorsal spino-cerebellar tract in the cat. IV. Synaptic connections of afferents from Golgi tendon organs and muscle spindles. *Acta physiol. scand.* **38**, 53–75.

LUNDBERG, A. & OSCARSSON, O. (1959). Identification of a third subdivision of the dorsal spino-cerebellar tract. *Experientia*, **15**, 195.

MCINTYRE, A. K. (1953). Synaptic function and learning. *Abstr. XIX int. physiol. Congr.* 107–114.

MCINTYRE, A. K., BRADLEY, K. & BROCK, L. G. (1959). Responses of motoneurones undergoing chromatolysis. *J. gen. Physiol.* **42**, 931–958.

MCINTYRE, A. K., MARK, R. F. & STEINER, J. (1956). Multiple firing at central synapses. *Nature, Lond.*, **178**, 302–304.

MARK, R. F. & MCINTYRE, A. K. (1956). Responses of second-order afferent neurones in the cat spinal cord. *Proc. Univ. Otago med. Sch.* **34**, 18–19.

MARK, R. F. & STEINER, J. (1958). Cortical projection of impulses in myelinated cutaneous afferent nerve fibres of the cat. *J. Physiol.* **142**, 544–562.

MARSHALL, W. H. (1941). Observations on subcortical somatic sensory mechanisms of cats under nembutal anaesthesia. *J. Neurophysiol.* **4**, 25–43.

MATTHEWS, B. H. C. (1933). Nerve endings in mammalian muscle. *J. Physiol.* **78**, 1–53.

MORIN, F. (1955). A new spinal pathway for cutaneous impulses. *Amer. J. Physiol.* **183**, 245–252.

MOUNTCASTLE, V. B. & POWELL, T. P. S. (1959). Neural mechanisms subserving cutaneous sensibility, with special reference to the role of afferent inhibition in sensory perception and discrimination. *Johns Hopk. Hosp. Bull.* **105**, 201–232.

OSCARSSON, O. (1956). Functional organisation of the ventral spino-cerebellar tract in the cat. I. Electrophysiological identification of the tract. *Acta physiol. scand.* **38**, 145–165.

OSCARSSON, O. (1958). Further observations on ascending spinal tracts activated from muscle, joint and skin nerves. *Arch. ital. Biol.* **96**, 199–215.

RENSHAW, B. (1946). Central effects of centripetal impulses in axons of spinal ventral roots. *J. Neurophysiol.* **9**, 191–204.

ROSE, J. E. & MOUNTCASTLE, V. B. (1954). Activity of single neurones in the tactile thalamic region of the cat in response to a transient peripheral stimulus. *Johns Hopk. Hosp. Bull.* **94**, 238–282.

RUDIN, D. O. & EISENMAN, G. (1951). A method for dissection and electrical study *in vitro* of mammalian central nervous tissue. *Science*, **114**, 300–302.

SZENTÁGOTHAI, J. & ALBERT, A. (1955). The synaptology of Clarke's column. *Acta morph. Hung.* **5**, 43–51.

THERMAN, P. O. (1941). Transmission of impulses through the Burdach nucleus. *J. Neurophysiol.* **4**, 153–166.

WALL, P. D. (1959). Repetitive discharge of neurons. *J. Neurophysiol.* **22**, 305–320.

J. Physiol. (1960), **153**, *pp.* 331–349
With 1 *plate and* 7 *text-figures*
Printed in Great Britain

FUNCTIONAL ORGANIZATION IN NUCLEUS GRACILIS OF THE CAT

BY G. GORDON AND C. H. PAINE
From the University Laboratory of Physiology, Oxford

(*Received* 25 *March* 1960)

The dorsal column nuclei (nucleus cuneatus and nucleus gracilis), receiving many primary afferent fibres from the dorsal columns and sending many secondary fibres into the medial lemniscus, might be expected to be interesting in that they could contribute to the central analysis of sensory information. These nuclei appear to have a high factor of safety in transmission, as judged by the work of Therman (1941), who studied their function as 'relays'. More recently, it has been suggested that they contain paths that are not purely monosynaptic and that their organization is therefore more complex than that of simple relays; and limited spatial facilitation and inhibition of single neurones have been described (Amassian & de Vito, 1957).

We have approached this problem from the point of view of the way or ways in which impulses set up by combinations of tactile stimuli are handled by these nuclei, bearing in mind that such phenomena as mutual spatial inhibition have been seen in other primary sensory nuclei—in the visual and auditory pathways, for instance—and are likely to be relevant in sensory discrimination. We have therefore been concerned particularly with the cutaneous receptive areas of the cells and with spatial interactions, whether facilitatory or inhibitory, upon these cells. We have largely confined ourselves to those cells in nucleus gracilis which responded to tactile stimulation of the hind foot. A preliminary communication on this subject has already been made (Gordon & Paine, 1959).

METHODS

Preparation and fixation of animals. Cats were used for all the experiments. The great majority were anaesthetized with sodium pentobarbitone, the initial dose given intraperitoneally (38 mg/kg) and subsequent small doses of about 10 mg given by intravenous cannula. Anaesthesia was maintained at a level sufficient to prevent any spontaneous movement of the animal or movement in response to stimulation. A few animals were decerebrated under ether by blunt section at intercollicular level; and at some stage after recovery from the anaesthetic it was always necessary to prevent movement in these animals, e.g. by giving 1 mg doses of decamethonium iodide intravenously, respiration being maintained with a pump.

Access to the dorsal-column nuclei was obtained by removing the arch of the atlas vertebra and the upper half of that of the axis and some of the skull overlying the posterior part of the cerebellum. It was also necessary to remove that part of the cerebellum which overlies the upper part of nucleus gracilis (Pl. 1). This was done by suction, and bleeding was controlled with gelatin sponge.

In about half the experiments cutaneous nerves in the hind limb were prepared for electrical stimulation and recording. Two nerve branches were used in each of these experiments, usually branches of the saphenous nerve or of the medial plantar nerve. No more of each nerve was dissected than was required for the application of the electrodes. The nerves were afterwards protected by immersion in light liquid paraffin at 37–39° C.

The head was clamped in an animal-holding frame, which has been described previously (Gaze & Gordon, 1954), with the internal auditory meatus and the hard palate as fixation points. The neck was held as rigidly as possible by a clamp applied to the sides of one of the lower cervical vertebrae.

In experiments involving electrical stimulation of the saphenous nerve, the hind limb was held by clamps applied to the knee and ankle. When plantar nerves were used, a drill driven through the calcaneum was clamped to a Perspex plate to which the skin edges were also tied.

The animal was kept warm by a 150 W infra-red heating lamp.

Recording from nucleus gracilis. Micropipettes, filled with 3 M-KCl, and with a resistance at 50 c/s of 1·5–5 MΩ, were used for recording from single neural units. The micropipette was connected to one grid of a DC push–pull cathode-follower mounted on the manipulator of the animal stand. A silver indifferent electrode was placed in the neck muscles and connected to the other grid of the input circuit, which was earthed. Output signals from the cathode-follower were amplified and displayed on an oscilloscope, low frequencies being attenuated by RC coupling. The over-all frequency band width (to 3 db. down) was 80 c/s to 10 kc/s, with a 5 MΩ electrode.

Vertical movement of the micropipette was controlled by an oil-filled micromanipulator, and insertion was observed under a binocular microscope. The toughness of the pia-arachnoid, particularly over the lower part of nucleus gracilis, often made it necessary to tear away small parts of this membrane with watchmakers' forceps in order to allow the electrode to penetrate without breakage or indentation. The area of brain and spinal cord exposed had the appearance shown in Pl. 1. The shallow groove seen on either side of the mid line was shown by repeated exploration to correspond to the lateral border of nucleus gracilis in its upper part and of funiculus gracilis more caudally. Above the obex the nucleus bends laterally and continues for some 2–3 mm along the caudal and dorsal lip of the fourth ventricle. The surface of this nucleus is covered by blood vessels of very variable distribution, many of them large; and it should be emphasized that the pattern of vessels in any particular animal largely determined which parts of the nucleus were available for exploration (see Pl. 1).

By far the greatest difficulty in these experiments was caused by vascular and respiratory pulsation in the spinal cord, which often made it impossible to obtain stable records from single cells. No reliable method was found for overcoming this pulsation, but matters were always improved by introducing the electrode through an opening in a shaped Perspex plate pressing lightly on the surface of the cord; and this method was used in about a third of the experiments. In a few particularly difficult cases an increase in the depth of anaesthesia was used as a last resort.

Orthodromic stimulation. In experiments involving the stimulation of cutaneous nerves a pair of silver hooks (2 mm separation) was placed on each nerve branch. Each pair was coupled by a 1:1 isolating transformer to the output of a square-pulse stimulator (Kay, Phillips & Teal, 1958). The pulses used were of 60 μsec duration. A pair of recording electrodes placed proximally, either on the parent nerve or on each of the branches, acted as a check on the effectiveness of the stimuli and of the absence of repetitive responses.

In other experiments the skin was stimulated by means of electro-mechanical transducers ('Advance' V1 moving-coil Vibration Generator). Each of these was driven by a separate power amplifier, triggered by square pulses of 4 msec duration from the stimulators mentioned above, and they were entirely independent in their action. The maximum excursion was about 2 mm, and the driving power was sufficient to keep the excursion the same irrespective of any load likely to be applied in skin stimulation. The wave form so produced was complex, containing two substantial waves each of 10 msec duration, the first larger than the second in the ratio of 3:1, and separated in time by 23 msec. Although not entirely satisfactory, this system will give only one effective stimulus for each electrical pulse when operated near threshold. Insulated rods attached to these transducers were applied to the skin surface or to cutaneous hairs.

Antidromic stimulation. A few preliminary attempts were made to activate cells in nucleus gracilis antidromically by applying stimuli to the region of the medial lemniscus at the caudal end of the thalamus. A manipulator aligned on Horsley–Clarke co-ordinates was used to drive a recording electrode (a steel needle insulated up to its tip) into the appropriate region. This electrode was finally fixed in a position from which the most satisfactory response to cutaneous stimulation of the hind limb was obtained. In frontal plane 5 this usually lay 5–6 mm lateral to the mid line. A pair of stimulating electrodes (steel needles insulated up to 1 mm from the tip) was then placed in position, one each side of the recording electrode, by sliding them into pre-set guides. The final position of the stimulating electrodes was such that they were about 1·5–2 mm apart with their tips about 0·5 mm deeper than that of the recording electrode. The guides were arranged so that the medial stimulating electrode (cathode) was slightly posterior and the lateral electrode (anode) correspondingly anterior to the recording electrode. The positions of the electrodes were afterwards determined histologically, by the method previously described (Gaze & Gordon, 1954).

Measurement and notation. In defining the position of any response in the rostro-caudal dimension of nucleus gracilis, the rostral end of the nucleus is taken as zero and the position given in mm caudal to this (Pl. 1). On this scale the obex position is about 2·2 mm, and is the point of reference from which many such positions were determined during experiments. In making these measurements no account was taken of the lateral curvature of the upper part of the nucleus. This curvature is exaggerated in Pl. 1 by the camera: the upper part usually makes an angle of 35–40° with the mid line.

The sizes of cutaneous receptive fields for single cells in nucleus gracilis are expressed here in cm^2. Such figures are approximate, and are based on a simple system of anatomical classification used during the actual experiments.

RESULTS

Nature of responses

Neural units responding to light mechanical stimulation of skin or hairs greatly outnumbered other types of unit in nucleus gracilis, those responding to stimulation of deep tissues such as joints forming some 10% of the units observed. The cutaneous units formed a fairly homogeneous group, in the sense that many of them were spontaneously active, that they responded repetitively to single stimuli applied to nerves or to mechanical stimuli applied to the skin, and that these responses, recorded extracellularly, were made up of spikes which were either almost purely negative or negative with a preceding positive phase. The recorded size of the spikes lay between 0·2 and 2 mV. Prolonged high-frequency injury

discharges were regularly found, and were associated with lack of further responses from the unit in question. Responses showing these features are typical of cellular regions; we consider that they were likely to have been recorded from cells rather than fibres, and we propose to refer to the responding units as cells in the course of this paper. Exploration of the dorsal column at C4–C5, a region known to be cell-free, gave a very contrasting picture, in which spontaneous activity was absent, extracellularly recorded spikes very small (0·2–0·3 mV max.) and predominantly positive, and injury discharges either very brief or absent.

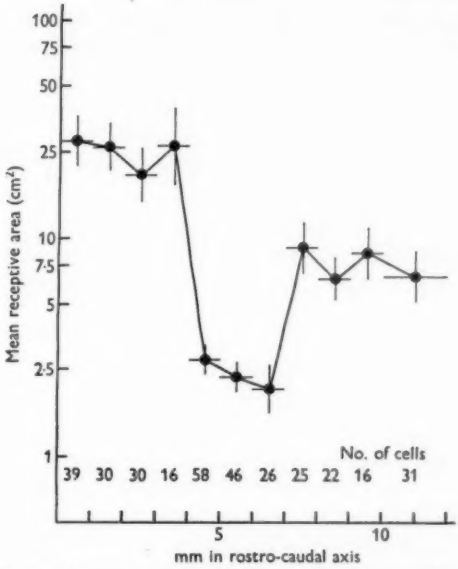
The number of responding cells was not uniform throughout the longitudinal axis of the nucleus, responses of 'cellular' type becoming increasingly scarce towards the caudal part (caudal to 10, in the scale in Pl. 1). This agrees with the decrease of cell density seen in histological sections, where scattered cells are observed down to the second cervical segment. In the upper part of the nucleus responding cells were again scarce, though this seemed likely to depend in part on factors other than cell density. These factors, which may include the amount of anaesthetic given and the adequacy of blood supply, were not very carefully investigated; but it was quite a common experience to find that the activity in the nucleus, in its upper part particularly, deteriorated after the experiment had gone on for some hours. It was also found that whereas most cells responded precisely and repeatably to skin stimulation after the manner of cutaneous nerve fibres, some cells in the upper part responded less consistently to such stimulation, particularly to stimuli repeated at short intervals.

Anatomical evidence has established an orderly segmental arrangement in the transverse axis of the dorsal column and dorsal-column nuclei, with the most caudal segments projecting dorsomedially (see Glees, Livingston & Soler, 1951). We have not precisely investigated this question, but our observations are completely consistent with previous work. It is also consistent with the above evidence that responses from increasingly proximal segments were often recorded with increasing depth of penetration. We have always recorded the depth from the surface at which each response was obtained, though such measurements were of variable accuracy according to the presence and amount of any indentation of the pia by the electrode. In cases where no indentation occurred we have satisfied ourselves that all cutaneous responses from the hind limb were recorded at depths well within the anatomical confines of the nucleus.

Receptive areas

The receptive areas of cells responding to tactile stimulation of the hind foot below the ankle were found to vary very widely, by a factor of 1000 or more. The smallest receptive area was less than 0·5 cm^2 on a pad or area

of hairy skin, and the largest consistently found included the whole of the ipsilateral hind limb and the lower half of the ipsilateral trunk. One cell was found whose receptive area included the skin of the whole body and all four limbs, but not the face. This cell was situated very near the surface within 1 mm of the rostral end of the nucleus; and it was found that cells with very large receptive areas tended to be concentrated in this region. For example, the receptive areas of 34 % of cells in the most rostral 4 mm of the nucleus extended beyond the foot on to the leg, and of 16 % on to the trunk (115 cells in all). It should be pointed out that these large



Text-fig. 1. Mean cutaneous receptive areas (A) of single cells responding to stimulation of the hind foot, plotted in relation to positions in the rostro-caudal axis of nucleus gracilis from which their responses were recorded. Measurements of A were approximate: they are shown on a logarithmic ordinate scale, with the S.E. in log A indicated by vertical bars. Horizontal bars represent length of section of nucleus yielding the responses from which each mean figure was calculated. Means derived from the numbers of units shown.

receptive areas are far greater than those of cutaneous nerve fibres from the foot, even of the 'wide-receptive' fibres described by Maruhashi, Mizuguchi & Tasaki (1952). The cells with the most restricted areas tended to be concentrated in a region 5–7 mm caudal to the rostral end, where 35 % of receptive areas were confined to 1 cm² or less on the hind foot, and only 4 % extended outside the foot (104 cells in all). This relation of

receptive area to position of the cell in the rostro-caudal axis of the nucleus is expressed graphically in Text-fig. 1. (Our original data on this point (Gordon & Paine, 1959) have been expanded here by including further cells investigated with Mr W. A. Seed, to whom we express our indebtedness.) It will be seen that in the caudal part of the nucleus the areas were again somewhat larger, although never comparable with the largest seen at the rostral end.

These changes in receptive area with rostro-caudal position were not peculiar to those cells with distal receptive areas including the hind foot. In a population of thirty cells whose receptive areas were centred in the leg or thigh and did not include the foot, sampled fairly uniformly throughout the upper 12 mm of the nucleus, the same relationship was found to apply. The mean figures for receptive areas were all somewhat greater than for cells whose receptive areas included the hind foot.

In one experiment in which responses of normal type were recorded from nucleus gracilis, five electrode tracks, widely spaced in the rostro-caudal axis, were made in nucleus cuneatus. The results, although too few in number to lead by themselves to a valid conclusion, were entirely consistent with what has been said above about the receptive areas in nucleus gracilis; and it may therefore be suggested that nucleus cuneatus has a similar organization in this respect.

Spatial interaction

Spatial interaction of two afferent paths on single cells of nucleus gracilis was studied by using pairs of stimuli applied either as electrical stimuli to different cutaneous nerve branches, or as mechanical stimuli to different areas of skin. The use of electrical stimuli to nerve branches had the advantages of providing better controlled stimuli, precise timing of the stimulus and complete freedom from physical interaction between stimuli as a possible source of error. One disadvantage of electrical stimulation is that one is limited to an examination of cells whose receptive areas lie within the distribution of the particular branches chosen for dissection, and that the number of observations is therefore likely to be small. Another important fact about this method is that the receptive area of any particular cell is very unlikely to lie entirely within the peripheral distribution of one of the selected branches and not at all within that of the other, unless the branches are remote enough from each other to minimize the overlap in their skin fields. It follows that this may provide a satisfactory method of examining spatial facilitation within a receptive field and its immediate surroundings. But if it is the case that a cell is inhibited by stimuli applied just outside its receptive area, then it is always likely that a stimulus applied to either nerve branch will produce a mixture of

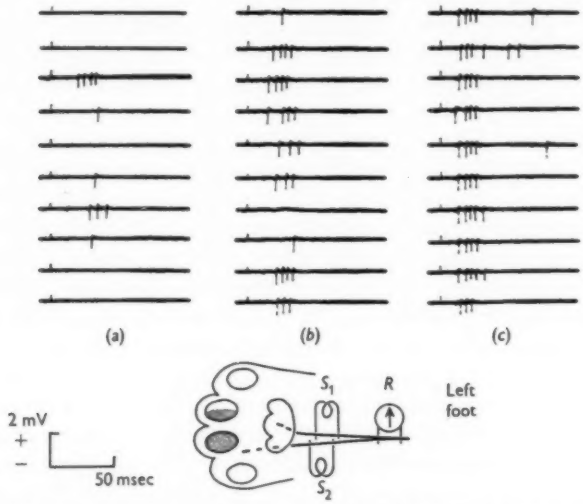
facilitation and inhibition whose exact proportions depend on the actual position of the receptive area within the joint distribution of the two branches. This difficulty can be largely overcome by the use of mechanical stimuli applied in known relation to the cutaneous receptive area; and this second method has in fact proved to be more useful in examining inhibitory interaction. Most of the observations described in this section were made on anaesthetized animals; but the experiments involving stimulation of nerve branches were repeated on decerebrate animals, with substantially the same results.

Spatial facilitation. Spatial facilitation was observed in the great majority of cases where the stimuli were applied to two nerve branches each contributing to the receptive area. In a group of 41 cells showing this effect and sampled fairly uniformly throughout the length of the nucleus, the receptive areas, all lying wholly or partially on the hind foot, ranged from 1 to 500 cm^2. Facilitation was also seen on two occasions where the receptive area was confined to the distribution of one branch, and the cell did not respond to stimulation of the other branch alone: the receptive areas of these two cells were each about 20 cm^2. The time course of facilitation was not investigated: usually a stimulus just above threshold was applied to each nerve branch in turn, and then the same stimuli were applied synchronously to both. Facilitation was recognized by an increase in the probablity of a response occurring, by shortening of latency, by an increase in the number of spikes fired in response to each stimulus or by a combination of these effects (see Text-fig. 2). It was clear that in almost all cells latency of response shortened with increasing spatial facilitation, and often to a very marked degree. It will be seen from Text-fig. 2, for instance, that the latencies for stimulation of each branch alone at threshold were 23 and 30 msec, whereas the latency with both stimuli together was 15 msec. Shortening of latency of a similar order was frequently seen when a stimulus to one nerve branch was increased from threshold to maximal; and as there was no repetitive response in the nerve, it is clear that this effect also depended on spatial facilitation—that is, on an increase in the number of active fibres. In such cases we have found the latency to shorten by 5–15 msec (35–100 % of the minimal latency). We have no evidence that this shortening of latency ever depended on the excitation of the δ fibre group in the nerve branch and on most occasions the record from the nerve itself proximal to the stimulating electrodes was satisfactory enough to show that no such excitation had occurred, even with stimuli maximal for the cells in question. Changes in latency of this order in a primary nucleus call for some further comment (see Discussion).

Mixed facilitatory and inhibitory effects on a single cell have been found on a number of occasions when a nerve was stimulated electrically. In

one clear instance weak single shocks given to a nerve branch supplying the receptive area (the centre pad of the foot) and its surroundings caused excitation, whereas strong shocks produced no response. Such effects are to be expected from the argument given earlier in this section.

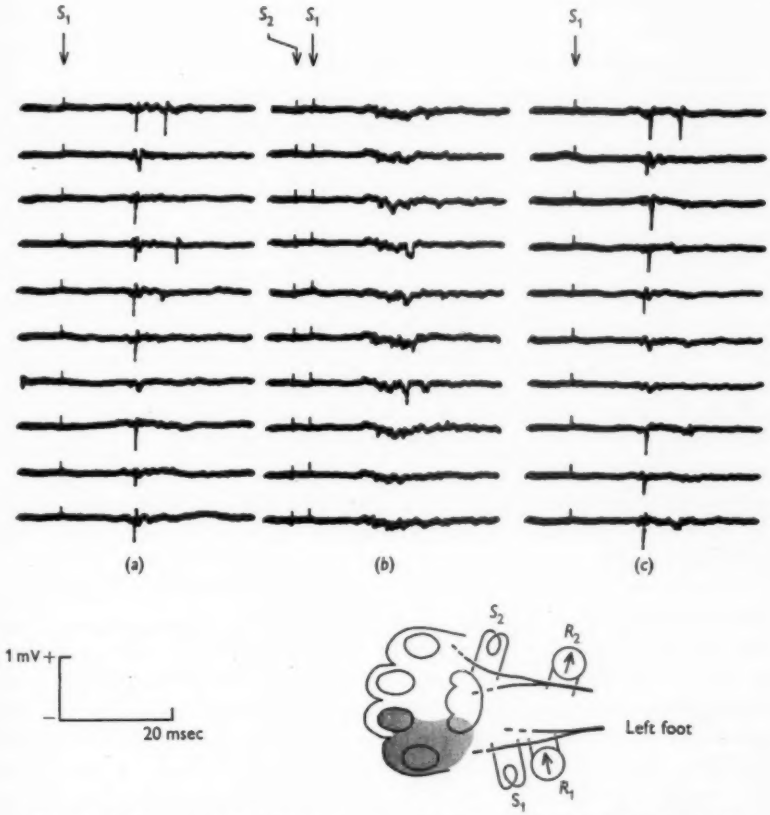
Spatial inhibition. In the course of experiments in which pairs of nerve branches were stimulated electrically, there were a number of instances in which a cell was fired by a test stimulus applied to one nerve and in which this response was reduced or abolished by a preceding conditioning



Text-fig. 2. Responses of a cell in nucleus gracilis (7–8 mm on A–P scale) to threshold electrical stimulation of two branches of the medial plantar nerve; sodium pentobarbitone anaesthesia. Each branch contributed to its skin receptive area (shaded in diagram, light touch), and spatial facilitation occurred when the two were synchronously stimulated. Each vertical column shows 10 successive sweeps at 1/sec. The electrodes *R* were used for monitoring the nerve action potential. (*a*) S_1 alone; response in 5/10 sweeps; mean latency 30 msec. (*b*) S_2 alone; response in 9/10 sweeps; mean latency 23 msec. (*c*) S_1 and S_2 synchronously; response in 10/10 sweeps; mean latency 15 msec.

stimulus to the other nerve (Text-fig. 3). Reduction of response in such cases was shown by a fall in the probability of a response, a smaller number of spikes, a lengthening of latency of response, or by combinations of these effects. The inherent limitations of this particular technique made it impossible to determine the form or distribution of skin stimulus necessary to elicit these inhibitory effects. No satisfactory series of observations was made on the time course of the inhibitory process, because recording conditions in these experiments were rarely stable enough to allow pro-

longed observation; but it was always our practice to determine roughly the interval by which the conditioning stimulus should precede the test stimulus to produce the greatest inhibition. With 10 such cells, with receptive areas in the lower leg or foot, this optimal interval varied between



Text-fig. 3. Responses of a cell in nucleus gracilis (6–7 mm on A–P scale) to threshold stimulation of a branch of the medial plantar nerve, showing inhibition by a conditioning stimulus applied to a branch of the lateral plantar nerve; sodium pentobarbitone anaesthesia. Its spontaneous discharge could be inhibited by light touch of ipsilateral leg, trunk or forelimb. The skin receptive area of this cell is shown shaded in the accompanying diagram (light touch). Each vertical column shows 10 successive sweeps at 1/sec. R_1 and R_2 were used for monitoring the nerve action potentials. (*a*) Test stimulus (S_1) only; response in 9/10 sweeps; mean latency 13·3 msec. (*b*) Conditioning stimulus (S_2) precedes S_1 by 3 msec; response in 1/10 sweeps; latency 17·0 msec. (*c*) S_1 only: a control repetition of (*a*) made immediately after (*b*); response in 8/10 sweeps; mean latency 13·5 msec.

zero and 15 msec, with a mean at 7·4 msec. No inhibitory effect was ever observed with the conditioning stimulus delivered later than the test stimulus.

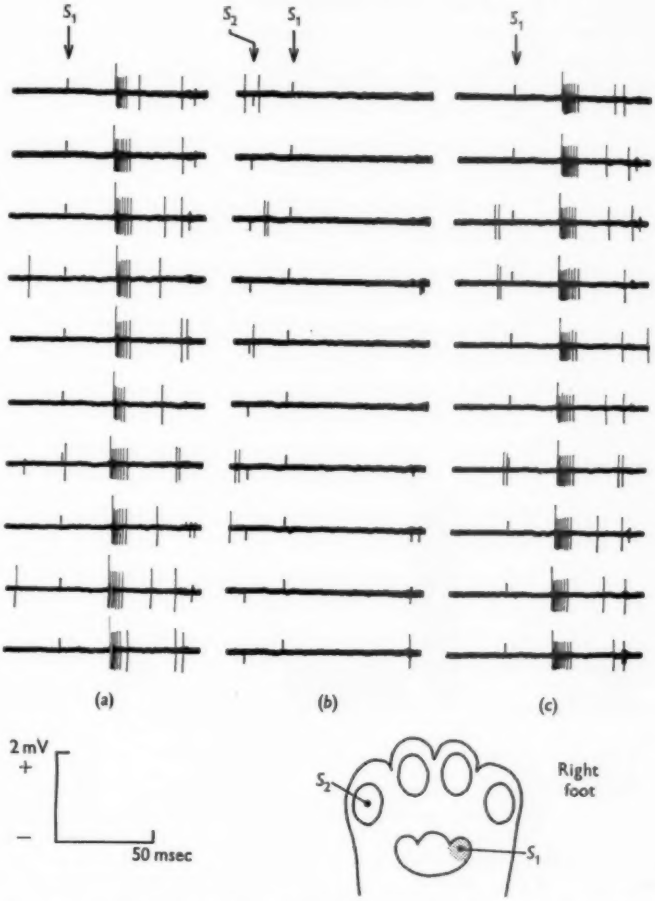
During the above experiments it was quite commonly found that cells which were spontaneously active could be inhibited by tactile stimuli applied outside their receptive areas, inhibition being seen as a temporary abolition of the spontaneous discharge; and it became clear that this inhibitory effect was very much more common than the experiments with nerve stimuli had suggested. In some cases it was clear that inhibition of spontaneous discharge was most effective with tactile stimuli immediately surrounding the receptive area and progressively less effective with stimuli further away up the leg. In other cases the inhibition could be produced with almost equal effectiveness by stimuli in widely separated parts of the ipsilateral surface of the body even including the forelimb.

This effect was investigated further by using pairs of mechanical stimulators (see Methods), one placed on the receptive area and the other on skin or hairs outside this area. This technique allowed a rough estimate to be made of the optimal time interval between conditioning and test stimuli, allowed investigation of cells which were not spontaneously active, and had certain advantages already mentioned in examining inhibitory interaction. Of nine cells satisfactorily investigated in this way, in which partial or complete inhibition was demonstrated, the optimal time interval, estimated roughly as before, lay between 0·5 and 20·5 msec with a mean at 10·4 msec. The conditioning stimulus always preceded the test stimulus. An example is shown in Text-fig. 4.

In six of these cases the test stimulus was applied to hairs only, and in the remaining three to a pad. In eight cases the conditioning stimulus was applied to a pad 1–2 cm away from the test stimulus, and in one case to one of the toes. The test and conditioning stimuli were just above threshold for their respective effects, and no stimulus applied to the skin was so large as to produce visible movements of any part of the foot. The stimulus amplitudes were of the order of 0·1 mm or less. It was hoped by spatial separation of the stimuli and by minimizing movement to avoid any artifact resulting from movement transmitted from one site of stimulation to the other.

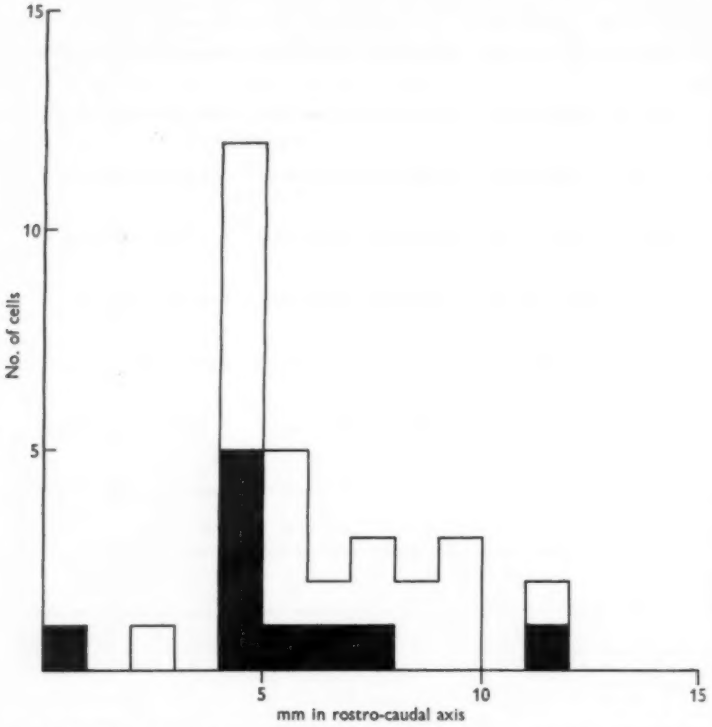
Relation of inhibition to receptive area and to position in nucleus. Altogether 31 cells were investigated whose receptive areas lay wholly or partially on the hind foot, and in which inhibition was demonstrated by one or other of the techniques described above. Of these, 16 were found by inhibition of spontaneous discharge alone, six by nerve stimulation and nine by the use of mechanical stimulators; and some in the two latter groups also showed inhibition of spontaneous discharge. This total figure derives largely

from experiments particularly designed to investigate inhibition, and should not be compared with the total number of cells investigated which can be derived from Text-fig. 6(*b*).



Text-fig. 4. Responses of a cell in nucleus gracilis (4·8 mm on A–P scale) to threshold mechanical stimuli on its receptive area (shaded in the accompanying diagram), showing inhibition of response by conditioning mechanical stimuli applied to another pad; sodium pentobarbitone anaesthesia. Each vertical column shows 10 successive sweeps at 1/sec; spikes retouched. (*a*) Test stimulus (S_1) only. (*b*) Conditioning stimulus (S_2) precedes S_1 by 20 msec; note abolition of response, and also absence of spontaneous activity between 10 and 90 msec after S_2. (*c*) S_1 only; a control repetition of (*a*) immediately after (*b*).

These cells showing inhibitory effects were distributed in the rostro-caudal axis of nucleus gracilis in the way shown in Text-fig. 5. It will be seen that the greatest number was found in the fifth millimetre caudal to the rostral border of the nucleus, and that they were mainly distributed at and caudal to this point. This distribution corresponds reasonably well



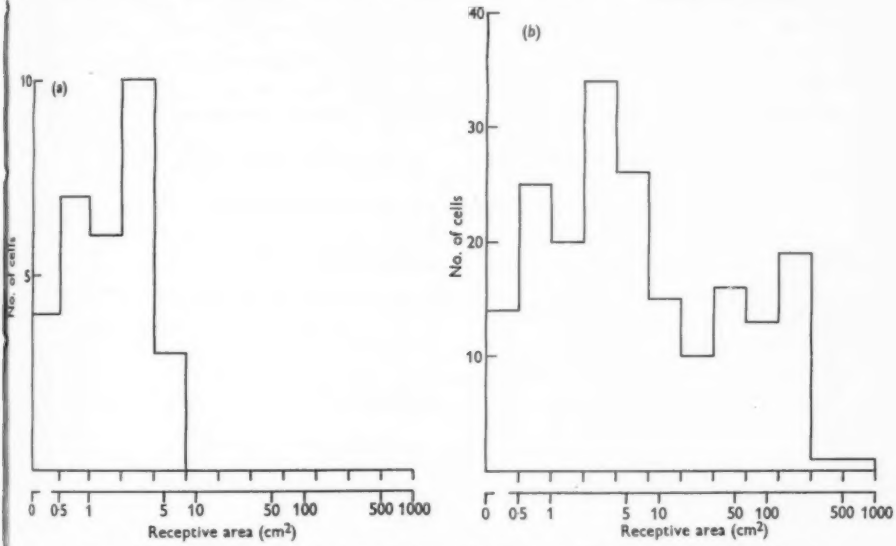
Text-fig. 5. Histogram showing numbers of cells which were inhibited by stimulation of skin or cutaneous nerves, related to their position in the rostro-caudal axis of nucleus gracilis. Position in 1 mm limits: zero on abscissa represents rostral border of nucleus. Receptive area of all cells lay wholly or partially on the hind foot. Black areas represent cells in which *complete* inhibition was observed.

with the trough in the graph of Text-fig. 1, which represents the region of the nucleus containing cells with the smallest receptive areas. This suggested relationship is further substantiated by Text-fig. 6(*a*), which shows the same group of cells related to the size of their excitatory cutaneous receptive fields. It will be seen here that they were fairly rigidly confined to the group of cells with small receptive areas, and that their distribution

within this group followed substantially the same pattern as that of the total population of cells investigated which had small receptive areas (compare Text-fig. 6(*a*) and (*b*)).

Antidromic stimulation

It seemed to be of great importance to determine the site or sites to which the cells in nucleus gracilis project, particularly when it had been found that the nucleus was not functionally homogeneous throughout its

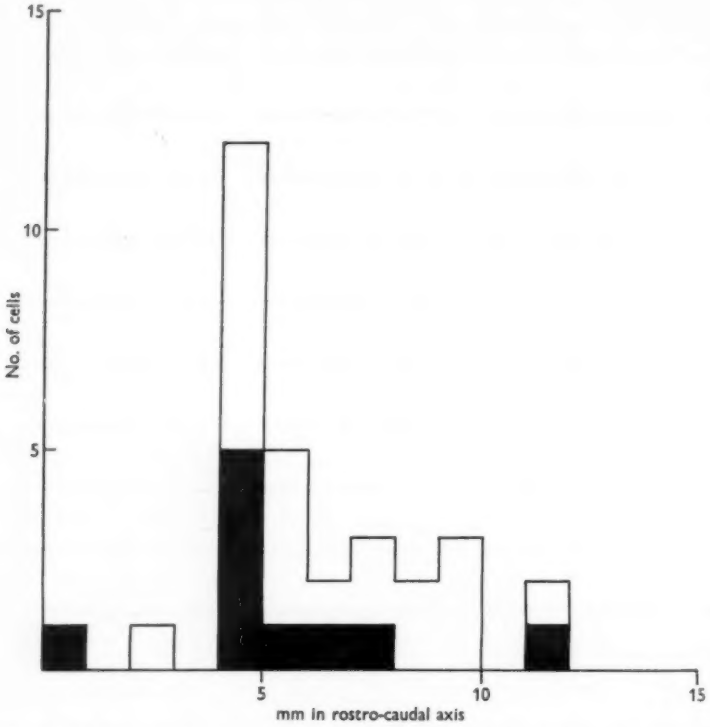


Text-fig. 6. Histograms relating numbers of cells investigated in nucleus gracilis to the sizes of their skin receptive areas. Receptive area indicated on a logarithmic scale (lower scale): histogram constructed on limits corresponding to successive doubling of receptive area (upper scale). Receptive areas of all cells lay wholly or partially on the hind foot. (*a*) Cells in which inhibition was observed (same cells as in Text-fig. 5). (*b*) Total cells investigated.

length. Antidromic stimulation of the cells, by applying electrical stimuli to expected sites of projection, provides one means of investigating this question. In the experiments described below, a preliminary attempt was made to determine the proportion of cells in various parts of nucleus gracilis which projected directly into the rostral part of the contralateral medial lemniscus and therefore into the thalamus.

The experimental technique has already been described (see Methods). Cells fired by stimulation through electrodes placed in the region of the medial lemniscus usually gave a single spike response at a latency of

These cells showing inhibitory effects were distributed in the rostro-caudal axis of nucleus gracilis in the way shown in Text-fig. 5. It will be seen that the greatest number was found in the fifth millimetre caudal to the rostral border of the nucleus, and that they were mainly distributed at and caudal to this point. This distribution corresponds reasonably well



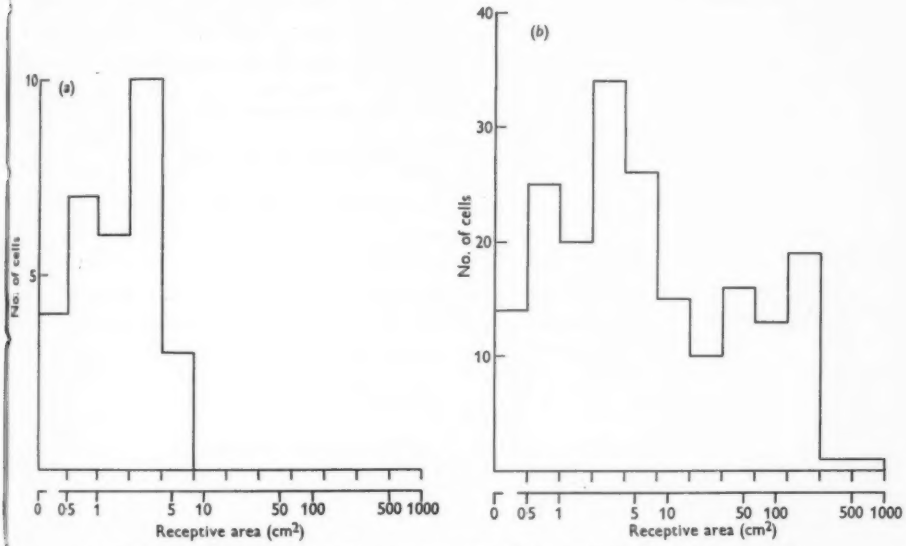
Text-fig. 5. Histogram showing numbers of cells which were inhibited by stimulation of skin or cutaneous nerves, related to their position in the rostro-caudal axis of nucleus gracilis. Position in 1 mm limits: zero on abscissa represents rostral border of nucleus. Receptive area of all cells lay wholly or partially on the hind foot. Black areas represent cells in which *complete* inhibition was observed.

with the trough in the graph of Text-fig. 1, which represents the region of the nucleus containing cells with the smallest receptive areas. This suggested relationship is further substantiated by Text-fig. 6(*a*), which shows the same group of cells related to the size of their excitatory cutaneous receptive fields. It will be seen here that they were fairly rigidly confined to the group of cells with small receptive areas, and that their distribution

within this group followed substantially the same pattern as that of the total population of cells investigated which had small receptive areas (compare Text-fig. 6(*a*) and (*b*)).

Antidromic stimulation

It seemed to be of great importance to determine the site or sites to which the cells in nucleus gracilis project, particularly when it had been found that the nucleus was not functionally homogeneous throughout its

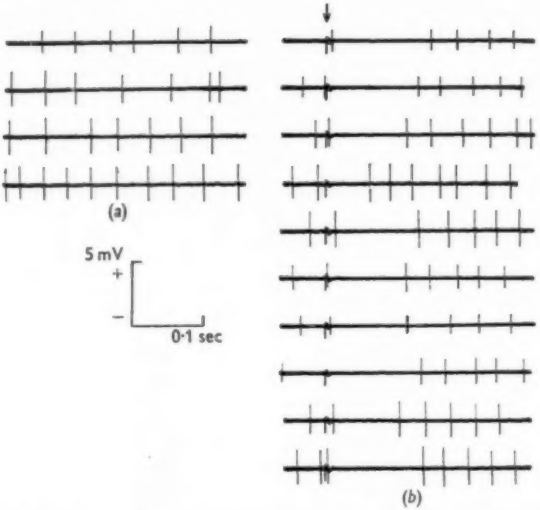


Text-fig. 6. Histograms relating numbers of cells investigated in nucleus gracilis to the sizes of their skin receptive areas. Receptive area indicated on a logarithmic scale (lower scale): histogram constructed on limits corresponding to successive doubling of receptive area (upper scale). Receptive areas of all cells lay wholly or partially on the hind foot. (*a*) Cells in which inhibition was observed (same cells as in Text-fig. 5). (*b*) Total cells investigated.

length. Antidromic stimulation of the cells, by applying electrical stimuli to expected sites of projection, provides one means of investigating this question. In the experiments described below, a preliminary attempt was made to determine the proportion of cells in various parts of nucleus gracilis which projected directly into the rostral part of the contralateral medial lemniscus and therefore into the thalamus.

The experimental technique has already been described (see Methods). Cells fired by stimulation through electrodes placed in the region of the medial lemniscus usually gave a single spike response at a latency of

1·5–2·5 msec, although some latencies were longer. In the group with short latencies the threshold and latency for any cell were remarkably constant, the latter varying by about 3% or approximately twice the measurement error. It was thought likely that the responses in this group were due to direct antidromic excitation. As a further test, each cell was normally subjected to tetanic stimulation with shocks just above threshold at 500/sec; and all the cells showing responses at short latency were found to follow this frequency. This was regarded as further evidence for antidromic excitation.



Text-fig. 7. Records from a cell in nucleus gracilis (3·8 mm on A–P scale), showing inhibition of spontaneous activity by single-shock stimuli applied to the region of the contralateral medial lemniscus; sodium pentobarbitone anaesthesia. From this position shocks of similar size elicited antidromic responses from a number of other cells in the nucleus. Skin receptive area of the cell: hairs on 16 cm² of the dorsolateral flank. Each vertical column shows a series of successive sweeps at 1/sec. Spikes retouched. (*a*) Cell firing spontaneously. (*b*) Activity conditioned by lemniscal stimuli (arrow indicates position of stimulus on sweep); stimulus 5 V, duration 60 μsec, stimulating electrodes 1·5 mm apart.

Little significance ought to be attached to negative results (i.e. failure to excite any particular cell in nucleus gracilis antidromically) in the small number of experiments carried out. It was found that the threshold voltages for antidromic excitation decreased and the proportion of positive results increased as the experimental technique was improved. In the two most satisfactory experiments we were satisfied by histological examination that the final millimetre (uninsulated) of the stimulating electrodes

lay close to the medial edge of the medial geniculate nucleus on their lateral side with their tips almost reaching the substantia nigra ventrally, so that they should have occupied the position usually ascribed to the medial lemniscus at this level (see Ranson & Ingram, 1932). In these two experiments 15 cells, or some three-quarters of the cells observed which had receptive areas including the hind foot, were fired antidromically. The thresholds varied between 0·7 and 13 V, with a mean at 5 V, using a 60 μsec square pulse. The cells in question were distributed fairly uniformly over the rostral 6 mm of the nucleus. They included some cells showing peripheral inhibitory interaction of the kind described earlier. These investigations have since been considerably extended with improved techniques, and it has been found that 80–90 % of the cells in the middle section of the nucleus can be fired antidromically from the contralateral medial lemniscus (Gordon & Seed, 1960).

It has been mentioned above that some responses to stimulation in the region of the caudal thalamus had much longer latencies (6–10 msec); and these were not considered to be antidromic responses because their threshold and latency were always variable and they sometimes included more than one spike. On one occasion the spontaneous discharge of a cell in nucleus gracilis was seen to be inhibited by single shocks applied to the caudal thalamus region (see Text-fig. 7); the threshold stimulus for this effect was 5 V with the electrodes in a position from which true antidromic responses of other cells were obtained at comparable thresholds.

DISCUSSION

The most striking facts which have emerged from this investigation concern the marked functional differentiation shown to exist in the rostrocaudal axis of this very elongated nucleus. The fact that many of the cells in the rostral 4 mm of the nucleus which responded to tactile stimulation of the ipsilateral hind foot had extremely large receptive areas indicates a very high degree of central convergence here. Cajal (1909) described a pre-terminal branch given off by many fibres of the gracile and cuneate funiculi of the mouse after entry into their respective nuclei, and ascending into their upper parts; and such a presynaptic collateral system provides one conceivable basis for wide convergence of the kind we have inferred, though convergence of post-synaptic axons or axon collaterals may also occur. Cajal (1896) also mentioned a few pre-terminal branches of fibres in funiculus gracilis crossing to the nucleus of the opposite side; and it is possible that such fibres, converging with those from the ipsilateral side, could be the basis of our otherwise surprising observation of one cell at the top of the nucleus whose receptive area extended over both sides of the body. Apart from noting the fact that the organization at the rostral

end of the nucleus provides a method of dealing with information derived from large areas of skin, it is probably not justifiable to speculate about its possible significance in central analysis. We have shown that cells of this kind project into the region of the medial lemniscus in the caudal thalamus; and it is now also known that some of them can be excited antidromically from the anterior lobe of the cerebellum (Gordon & Seed, 1960).

The very sharp contrast between the rostral and middle parts of the nucleus suggests different types of nuclear organization. The middle part, where receptive areas are small and mutual inhibition is common, seems well qualified to pass on information related to spatial discrimination; and we have shown that cells of this kind project from the middle part of the nucleus to the caudal thalamus. Limited inhibitory interaction has been observed in nucleus cuneatus of the cat by Amassian & de Vito (1957), who found—and this is also our impression—that these effects were relatively resistant to barbiturate anaesthesia. It is clear that we have found inhibitory effects to be much more common than they did and that we have ascribed much more importance to them. This apparent discrepancy is presumably the result of our finding that inhibitory interaction was effectively limited to a quite small part of the nucleus where it was very prominent; and we have given some reasons for believing that nucleus cuneatus is organized on similar lines to nucleus gracilis. Inhibitory interaction has also been observed in cortical cells responding to cutaneous stimulation in the cat by Mountcastle (1957), who found such inhibition to be rather uncommon in the population of cells he studied, and to be very easily abolished by small doses of barbiturate. This difference from our results as regards susceptibility to anaesthesia seems to indicate that further inhibitory mechanisms of a type highly sensitive to anaesthetic must exist in this sensory system. Mutual inhibitory interaction has now been observed in a number of sensory systems where its possible significance in increasing contrast by emphasizing discontinuities in a stimulus pattern has been recognized and evaluated (e.g. Whitfield, 1957; Hartline & Ratliff, 1958).

Our investigation contributes little or no information about the interrelationship of neurones within the nucleus, and the possibility mentioned by Amassian & de Vito (1957) that these nuclei might contain true internuncial cells. We have always applied the stimuli at too great a distance from the nucleus to make any assessment of the delays involved in nuclear transmission. Only one of our observations seems to bear on this question, and this was the very marked shortening of latency by 5–15 msec which was observed in some cases of spatial facilitation. The time scale of effective facilitation implied here seems to be considerably longer than would be expected from the known properties of monosynaptic systems (see Coombs,

Curtis & Eccles, 1956). The possibility also exists that this nucleus might contain inhibitory internuncial cells, in accordance with the system believed to exist in connexion with spinal motoneurones (Eccles, Fatt & Landgren, 1956). We have no evidence bearing on these possibilities; though if further evidence were to indicate the existence of a substantial group of cells which could not be excited antidromically, the question would then become worthy of further examination. The part of the nucleus about which we know least is its caudal end, where we have shown the receptive areas of the cells to be intermediate in size between those found in the upper and middle parts. We do not yet know to what extent these cells are accessible to antidromic excitation.

A further problem is presented by our finding excitatory and inhibitory effects upon cells in nucleus gracilis as a result of stimulating the region of the medial lemniscus. The excitatory effects were distinguishable from antidromic excitation by their long and variable latency and by other characteristics already mentioned; and a similar distinction was made by Amassian & de Vito (1957). It is impossible to explain such effects with the knowledge at our disposal, though two obvious hypotheses present themselves. One is that descending pathways to the nucleus were being stimulated; and the other that the effects were due to antidromic excitation of axons proceeding from the nucleus and giving rise there to axon collaterals, these collaterals either ending on the cell observed or affecting it through internuncials. We are inclined to prefer the second possibility, in view of there being other grounds for believing the stimulating electrodes to have been in or very near the medial lemniscus; and Amassian & de Vito (1957) also took this view. A tentative hypothesis has been put forward elsewhere (Gordon & Seed, 1960), based on acceptance of this idea and taking into account further information about the distribution of such 'trans-synaptic' effects in the nucleus. If our interpretation of the effects themselves could be substantiated, a method would certainly then exist for studying new aspects of the internal nuclear organization.

SUMMARY

1. Nucleus gracilis of the cat was investigated with a single-unit recording technique, attention being specially directed to those cells responding to tactile stimulation of the skin or hairs of the hind foot.

2. In a population of such cells, the skin receptive areas were found to vary by a factor of 1000 or more. It was established that receptive area was related to the position of the cell in the rostro-caudal axis of the nucleus, those with the largest areas lying most rostral. Six millimetres or so caudal to the rostral pole most cells had very small receptive areas. More caudally still the cells had intermediate receptive areas.

3. Spatial facilitation of many cells was easy to demonstrate by a technique involving electrical stimulation of pairs of skin nerves.

4. Spatial inhibition was frequently found among the cells with small receptive areas lying (in the rostro-caudal dimension) in the middle section of the nucleus, and was demonstrated by a variety of techniques. It was most often produced by electrical or mechanical stimuli applied near the receptive area but outside it. More distant stimuli—applied to the forelimb, for instance—were effective with some cells.

5. It was shown that cells of the kinds described above, and lying in the rostral and middle sections of the nucleus, may be fired antidromically by electrical stimuli applied to the region of the contralateral medial lemniscus in the caudal thalamus. Other cells were fired by these means under circumstances suggesting a trans-synaptic path; and one cell was inhibited.

6. It was concluded that nucleus gracilis shows a well marked functional differentiation in its longitudinal axis which deserves further investigation. Cells in two functionally distinct regions—the rostral and middle sections—project to the thalamus.

We should like to acknowledge much generous help and advice from Dr R. H. Kay. We are also indebted to Mr W. A. Seed for the photograph in Pl. 1.

REFERENCES

AMASSIAN, V. E. & DE VITO, J. L. (1957). La transmission dans le noyeau de Burdach. *Colloq. int. Cent. nat. Rech. sci.* **67**, 353–393.

CAJAL, S. R. (1896). *Beitrag zum Studium der Medulla Oblongata des Kleinhirns und des Ursprungs der Gehirnnerven*, pp. 50–51. Leipzig: Barth.

CAJAL, S. R. (1909). *Histologie du système nerveux de l'homme et des vertébrés*, pp. 896, 905. Paris: Maloine.

COOMBS, J. S., CURTIS, D. R. & ECCLES, J. C. (1956). Time courses of motoneuronal responses. *Nature, Lond.*, **178**, 1049–1050.

ECCLES, J. C., FATT, P. & LANDGREN, S. (1956). Central pathway for direct inhibitory action of impulses in largest afferent nerve fibres to muscle. *J. Neurophysiol.* **19**, 75–98.

GAZE, R. M. & GORDON, G. (1954). The representation of cutaneous sense in the thalamus of the cat and monkey. *Quart. J. exp. Physiol* **39**, 279–304.

GLEES, P., LIVINGSTON, R. B. & SOLER, J. (1951). Der intraspinale Verlauf und die Endigungen der sensorischen Wurzeln in den Nucleus gracilis und cuneatus. *Arch. Psychiat. u. Z. Neurol.* **187**, 190–204.

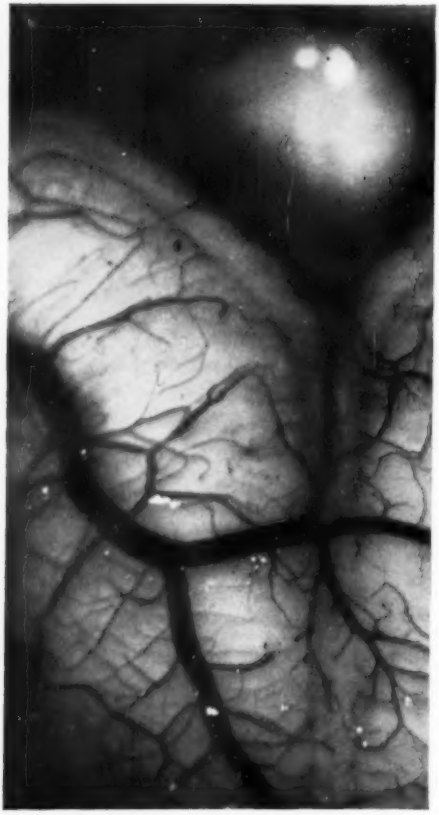
GORDON, G. & PAINE, C. H. (1959). Some features of the physiological organization of nucleus gracilis in the cat. *J. Physiol.* **148**, 34–35*P*.

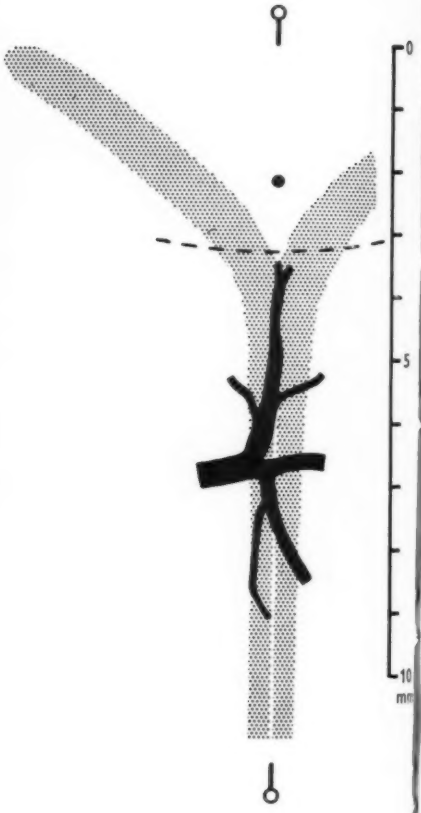
GORDON, G. & SEED, W. A. (1960). Responses of single neurones in nucleus gracilis of the cat to lemniscal and cerebellar stimulation. *J. Physiol.* **152**, 63*P*.

HARTLINE, H. K. & RATLIFF, F. (1958). Spatial summation of inhibitory influences in the eye of *Limulus*, and the mutual interaction of receptor units. *J. gen. Physiol.* **41**, 1049–1066.

KAY, R. H., PHILLIPS, C. G. & TEAL, R. H. (1958). A versatile stimulator. *Electron. Engng*, **30**, 575–578.

MARUHASHI, J., MIZUGUCHI, K. & TASAKI, I. (1952). Action currents in single afferent nerve fibres elicited by stimulation of the skin of the toad and the cat. *J. Physiol.* **117**, 129–151.





(*Facing p.* 349)

MOUNTCASTLE, V. B. (1957). Modality and topographic properties of single neurons of cat's somatic sensory cortex. *J. Neurophysiol.* **20**, 408–434.

RANSON, S. W. & INGRAM, W. R. (1932). The diencephalic course and termination of the medial lemniscus and the brachium conjunctivum. *J. comp. Neurol.* **56**, 257–275.

THERMAN, P. O. (1941). Transmission of impulses through the Burdach nucleus. *J. Neurophysiol.* **4**, 153–166.

WHITFIELD, I. C. (1957). The physiology of hearing. *Prog. Biophys.* **8**, 1–47.

EXPLANATION OF PLATE

PLATE 1

Photograph (left) of the dorsal aspect of a living cat's medulla and spinal cord at the level of the dorsal column nuclei. The caudal part of the cerebellum has been removed, exposing the 4th ventricle, which lies out of focus at the top. Nucleus gracilis is shown shaded in the accompanying key (on right), and zero on the millimetre scale corresponds to its rostral border. ○— —○, mid line; ●, obex; – – –, approximate position of caudal border of cerebellum before excision.

J. Physiol. (1960), **153**, *pp.* 350–357
With 3 text-figures
Printed in Great Britain

PERCEPTION OF LINEAR MOTION FOLLOWING UNILATERAL LABYRINTHECTOMY: VARIATION OF THRESHOLD ACCORDING TO THE ORIENTATION OF THE HEAD

By E. G. WALSH

From the Department of Physiology, University of Edinburgh

(*Received* 6 *April* 1960)

It has long been believed that the otolith organs are sensitive to gravity, to linear acceleration and to vibration, but the division of labour between the utricle and saccule has been a matter of dispute. One view is that the utricle alone is concerned with the sensitivity to the direction of the gravitational field and to linear acceleration. The saccule is thus looked upon as being solely a vibration receptor. Much of the evidence as it relates to lower vertebrates favours this interpretation (Lowenstein, 1936), and there is little relevant information for the human being. It was felt that a study of the responses of people who had lost the use of the inner ear on one side might have a bearing on this problem, for the elimination of two of the four otolith organs might simplify the analysis. Apparatus has been developed which subjects the person to controlled linear acceleration in the horizontal plane. Measurements have been made of the sensory threshold, the least acceleration that allows the person to determine the direction in which he has moved. Attention has been paid to the relationship between the orientation of the maculae of the otoliths and the threshold. A preliminary account of this work has already been published (Walsh, 1959).

METHODS

Initial observations made use of a trolley which was pulled along a railway of some 3 m length under the influence of falling weights or an electric motor. The apparatus finally evolved consisted of a stretcher which could be moved by the use of compressed air and which was supported from the ceiling by wires 4 m long (Fig. 1). The person lay blindfolded on a polyurethane (plastic) mattress. As the weight of the person was taken by the wires the static friction to movement was extremely low. To the underneath of the stretcher was fixed a jointed frame furnished with ball races. To the frame was attached a ram of 2·5 cm bore into which oil was driven from a reservoir by compressed air of up to 60 lb./sq. in. pressure (4·2 kg/cm^2). The consistency of the oil was such as to damp critically the oscillations of the system. By varying the pressure of the air the acceleration of the stretcher could be adjusted. The motion was recorded by an electromagnetic pick-up, the coil of which moved with the frame whilst the magnet was stationary. The electrical output after amplification was recorded by an ink-writing pen. The deflexion was proportional to the velocity

of the stretcher. The system was calibrated by detaching the ram from the frame and measuring the deflexions corresponding to known excursions of free oscillation.

The person was exposed by this technique to a brief (0·4 sec) period of acceleration followed by a much more prolonged period of more gradual deceleration. Near threshold the acceleration alone was sensed. The frame could be rotated, so that the motion was towards the head, towards the feet, or to either side. Threshold was taken as the least acceleration which would allow the person reliably to determine the direction in which he was moved.

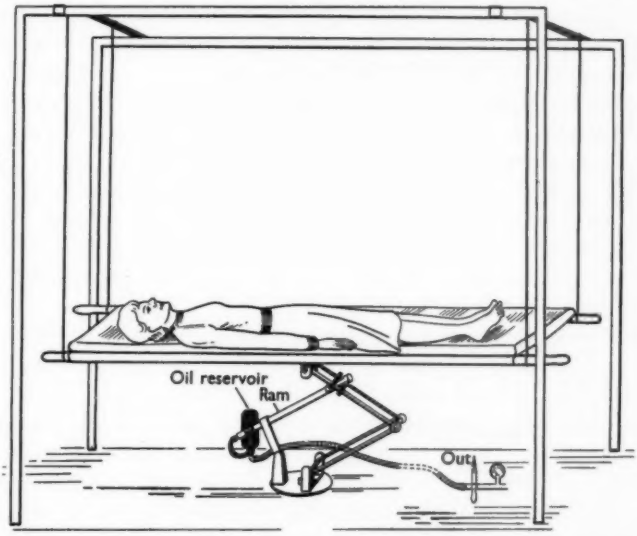


Fig. 1. Pneumatically controlled stretcher. Horizontal movement was initiated by connecting the airline through the tap to a reservoir of compressed air. As the stretcher moved, so the weight of the body opposed the motion to an ever increasing degree. The initial acceleration thus changed to a deceleration. The movement became slower and slower and was imperceptibly arrested.

Correct responses in each of the four cardinal directions were required. The movements of the stretcher were strictly speaking not linear but curvilinear. As the stretcher moved horizontally it also rose. This effect was negligibly small and did not involve any rotation, the stretcher always remaining parallel with the floor.

Use was also made of a parallel swing, an apparatus employed by Jongkees & Groen (1946). A stretcher was suspended at its four corners by wires 1·6 m long. With care, small transverse oscillations could be induced so that the person was carried alternately to the right and to the left. The movement was silent and free from unwanted vibrations.

RESULTS

Sensitivity when moving towards or away from damaged side

Quix (1925) believed that in the human being the saccule is sensitive to movement towards its own side, a situation in which the pressure of the membrane on the epithelium is increased. Initial observations making

use of the railway failed to support this viewpoint. Nine patients were tested, seven sitting, two lying. The thresholds towards and away from the side of the lesion were equal for six of the patients; for two the threshold was higher on moving to the side of the lesion; for one higher on moving to the normal side. As it was considered important to check these findings the problem was approached in another way. It was felt that small differences in sensitivity might be more readily detected if the person was subjected to rhythmic to-and-fro movement. Close to threshold they might be expected to note that movement towards one side was felt more strongly than movement in the opposite sense. Use was then made of the parallel swing to provide small transverse movements. Eight patients with unilateral labyrinthine lesions were investigated in the supine position. They reported that the movements were equally clear in both directions. Finally, the data obtained with the pneumatically controlled stretcher may be considered. Six subjects were tested when lying supine on the stretcher as to their sensitivity for movements to the right and left. No constant differences could be found.

These various methods thus agree in showing no consistent differences in sensitivity on moving towards or away from the side of the lesion when the person is lying on his back. The finding was unexpected, in view of the report by Jongkees (1950) that in the rabbit the removal of a saccule gives rise to insensitivity to movements directed across the head towards the damaged side. The scheme put forward by Quix (1925) is clearly untenable for the human being if the vestibular apparatus is responsible for the responses. It is hoped later to publish control data showing the role of the vestibular apparatus in the perception of movements on the parallel swing. The importance of the vestibular apparatus for the observations made with the pneumatically controlled stretcher is established by the data in the following section.

Variation in threshold with position of the head in space

In six subjects tested on the pneumatically controlled stretcher a variation in sensitivity was found according to the position of the head. When the person lay on the side of the lesion the threshold was consistently higher than when he lay on the normal side. The results are shown in Table 1. Of each pair of figures, that obtained with the person lying on the side of the lesion is the higher. The chance of such an occurrence being due to the operation of chance alone is 1 in 128. The difference is clearly of statistical significance although it varies very considerably in magnitude.

Five of the people were hospital subjects, the sixth was a 20-year-old female medical student (Miss B.) who had been deaf in one ear since infancy. Caloric tests on Miss B. revealed a minimal degree of nystagmus with hot

water and no response at all to cold on repeated testing; the responses on the other side were normal. The results of a number of tests on the pneumatically controlled stretcher are summarized in Fig. 2. The numbers correspond to thresholds (cm/sec/sec) at the corresponding position of the head. Two sets of numbers alongside a figure of the head show that the test was performed twice in that position and the results indicate the repeatability of the method under favourable circumstances. When the damaged

TABLE 1. Unilateral loss of vestibular function

Sex	Age		Threshold (cm/sec/sec) Lying on sound side	Lying on side of lesion
F	20	L. nerve deafness from infancy	12 10·5	20 13
M	41	R. labyrinthectomy	13	14
M	51	L. labyrinthectomy	31	59
M	54	R. labyrinthectomy	26	35
M	55	L. labyrinthectomy	15	22
F	59	R. acoustic neuroma	32	41
		Means	19·9	29·1

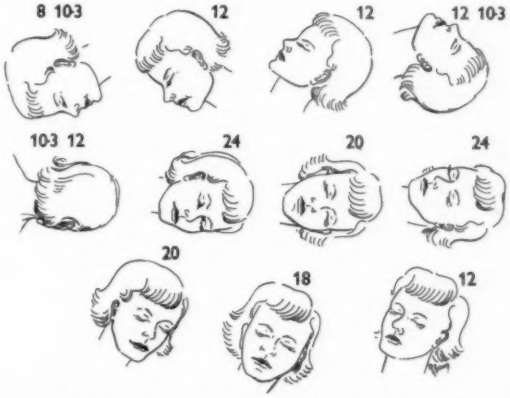


Fig. 2. Change of threshold with orientation of head in subject with left-sided nerve deafness (Miss B.). Head is viewed from side of stretcher, and numbers correspond to thresholds (cm/sec/sec).

Top row; alteration of position of head in sagittal plane caused little variation in threshold.

Middle row; subject lying on her side. Threshold was elevated when she lay on her left side (2nd, 3rd and 4th figures, in which she faces 45° downwards, horizontally and 45° upwards, respectively).

Bottom row; subject lying on her left side but with her head propped up and neck flexed towards her right shoulder. Threshold was elevated in the first two positions (facing 45° downwards and facing straight forwards), but not in the third (facing 45° upwards).

side was downwards the threshold was consistently elevated, a clear confirmation of the finding obtained with the other subjects. In this position she experienced difficulty also in describing the exact orientation of her head. Nevertheless, she usually lay in that position when in bed, for otherwise hearing was not satisfactory, the sound ear being obstructed by the pillow.

The relative positions of the main parts of the otolith maculae are shown in Fig. 3 (cf. Camis, 1930). According to Corvera, Hallpike & Schuster (1958) the two saccules lie almost parallel, occupying sagittal planes,

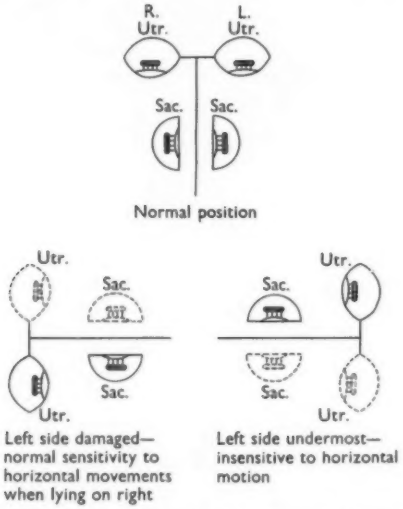


Fig. 3. Relative positions of saccule and utricle. In each figure the head is viewed from in front (after von Holst (1949) and Birukow (1959)).

whilst the two utricles are almost horizontal when the head is tilted a little forward. The anterior parts of the utricular maculae and the dorsal lobes of the saccular maculae are described as being angulated with respect to the main parts of the receptive epithelium. The existence of these small zones may partially mask alterations of sensitivity with position that would otherwise be more striking.

The systematic change in threshold with position described above clearly indicates that the vestibular apparatus is concerned in the responses. Movement may, however, stimulate also the skin. Even if the vestibular apparatus was completely insensitive the motion might still be detected as a result of the pressure on the trunk resulting from the acceleration of the stretcher. The alteration in the sensitivity of the vestibular apparatus

according to its orientation may thus be greater than indicated by the numerical data.

When a person lies on one side the maculae of the utricles lie close to a vertical plane at right angles to the long axis of the body. A shearing force will thus result from horizontal movements in a transverse direction, pressure or traction from movements to the head or to the feet. To ascertain how sensitive the utricle might be under these circumstances the proportion of correct responses obtained in experiments with the patient lying on the side of the lesion has been investigated. The data for the six patients of Table 1 have been taken, and are analysed in Table 2. The responses are those to stimuli close to threshold. Calculation of χ^2 gave a value of 2·9, which for 3 degrees of freedom is not significant. Evidently the utricle is insensitive to horizontal movement when its membrane is vertical. When the patient lay with the normal side downwards his sensitivity in the four directions of motion appeared to be equal. The value for χ^2 is 0·6, which is not significant.

TABLE 2. Proportion of correct responses for movement in different directions

	Movement to head		Movement to feet		Movement forwards		Movement backwards		
	No. of trials	No. correct	No. of trials	No. correct	No. of trials	No. correct	No. of trials	No. correct	χ^2
Lying on labyrinthectomized side	32	16	22	15	26	7	34	11	2·9
Lying on sound side	31	13	28	15	27	13	24	12	0·6

DISCUSSION

When a patient with a unilateral lesion lies on his normal side the membrane of the remaining utricle lies approximately in a vertical plane at right angles to the long axis of the stretcher. It is inconceivable, whatever view is taken as to the adequate stimulus, that an otolith organ would show the same sensitivity to a shearing force as to a force directed perpendicular to the macula; yet the patient has the same sensitivity for movements to the head or to the feet as to movements forwards or backwards. This finding suggests that the saccule participates in the responses. The change of threshold of the patients with unilateral labyrinthine lesions, according to their posture, also suggests that the saccules are concerned in the responses to linear acceleration. As the maculae of the utricles lie in almost the same plane it is difficult to see why this asymmetry should occur if they alone were concerned in the responses. The results would correspond to the right saccule being used to signal horizontal movements when lying on the right, the left saccule being involved when

lying on the left. It is implicit in this scheme that the saccule is sensitive to a shearing force but that the sensitivity varies according to the direction of the steady pull on the membrane due to gravity. Such a concept indicates that both traction and shearing forces must be considered; the need for an extension and synthesis of earlier views appears inescapable.

The macula is evidently sensitive to acceleration in its own plane, and can give information as to the direction of action of force in that plane. When the saccular membrane lies above its macula, gravity will tend to pull it to one side or to the other. This mechanical consideration may explain the poor sensitivity to horizontal movement in that position. With the membrane in a dependent position it comes to lie close to its mid position and maximal sensitivity is shown. The importance of a biasing force to one side will vary with the tightness of the suspension of the membrane. The results afford a suggestion that the utricle behaves in a similar way, for it appears to be insensitive to horizontal movement when its membrane is in a vertical plane (p. 355). In this position the shearing effect of gravity will be maximal; the membrane will be strongly pulled from its position of rest.

As an analogy a bell may be considered, for it can be rung by horizontal movements easily when in its normal position, with difficulty when inverted and not at all when on its side. The results harmonize with those obtained by Hasegawa (1938) on studying the frog after denervation of the saccules. The reflex responses to vertical jolting were lost. In Hasegawa's experiments the shearing force acted with or against gravity and not at right angles as in the experiments described in the present paper. On the analogy with the bell Hasegawa's results would have been predicted, for to be rung effectively by being rapidly raised and lowered a bell must be held horizontal.

SUMMARY

1. A suspended stretcher moved by compressed air has been used to test the sensibility of the human subject to linear acceleration. The person was required to ascertain the direction in which the motion had occurred, and the least acceleration needed was measured. Patients were investigated who had lost the use of the vestibular apparatus on one side.

2. When the patient lay on his back the sensitivity to movement towards and away from the damaged side appeared to be the same.

3. The sensitivity was consistently reduced when the patient lay with the damaged side downwards.

4. It is argued that these results indicate that the saccule partakes in the responses and that it is responsive to a shearing force parallel to the main part of its macula. It is concluded also that the direction of the gravitational bias is important in determining the effectiveness of a given

shearing force. Greater sensitivity to horizontal motion results when the saccular otoliths hang than when they press on the macula.

The author wishes to thank Professor N. M. Dott and Mr I. Simson-Hall for access to patients in their departments of the Royal Infirmary, Edinburgh; Drs I. Simpson and J. Stanton for their co-operation in referring patients to him for these investigations; and C. Shepley, Esq., Department of Medical Illustration, for the figures. The apparatus was constructed by G. Wright.

REFERENCES

BIRUKOW, G. (1959). *Statischer Sinn* in *Handbuch der Zoologie*, ed. HELMCKE, J. G., VON LENGERKEN, H. and STARCK, D. Band 8, Teil 4, pp. 1–40. Berlin: Gruyter.

CAMIS, M. (1930). *The Physiology of the Vestibular Apparatus*. Translated by CREED, R. S. Oxford: Clarendon Press.

COZVERA, J., HALLPIKE, C. S. & SCHUSTER, E. H. J. (1958). A new method for the anatomical reconstruction of the human macular planes. *Acta oto-laryng., Stockh.*, **49**, 4–16.

HASEGAWA, T. (1938). Die stato-kinetische Funktion des Saccules. *Z. Hals-, Nas.-u. Ohrenheilk.* **43**, 129–132.

JONGKEES, L. B. W. (1950). On the function of the saccule. *Acta oto-laryng., Stockh.*, **38**, 18–26.

JONGKEES, L. B. W. & GROEN, J. J. (1946). The nature of the vestibular stimulus. *J. Laryng.* **61**, 529–541.

LOWENSTEIN, O. (1936). The equilibrium function of the vertebrate labyrinth. *Biol. Rev.* **11**, 113–145.

QUIX, F. H. (1925). The function of the vestibular organ and the clinical examination of the otolith apparatus. *J. Laryng.* **40**, 425–443.

VON HOLST, E. (1949). Zur Funktion des Statolithenapparates im Wirbeltier-labyrinth. *Naturwissenschaften*, **36**, 127–128.

WALSH, E. G. (1959). Sensibility of patients to linear acceleration after unilateral labyrinthectomy. *J. Physiol.* **149**, 33*P*.

J. Physiol. (1960), **153**, *pp.* 358–369
Printed in Great Britain

SODIUM AND POTASSIUM MOVEMENTS IN KIDNEY CORTEX SLICES FROM NEW-BORN ANIMALS

By R. WHITTAM

From the Medical Research Council Cell Metabolism Research Unit, Department of Biochemistry, University of Oxford

(*Received* 21 *April* 1960)

Slices of the cortex cut from kidneys of adult rabbits, rats and guinea-pigs depend on respiration for the maintenance of high potassium and low sodium concentrations in the intracellular fluid, because a loss of potassium and a gain of sodium occur when respiration is inhibited by cooling, anoxia or cyanide (Mudge, 1951; Whittam & Davies, 1953; Aebi, 1953). The metabolism of kidney cortex slices from these adult animals is predominantly aerobic, with high rates of oxygen consumption and low rates of anaerobic glycolysis. Thus, in guinea-pig kidney cortex slices the energy supply anaerobically is only 1 % of that aerobically and is about one sixth of the minimum required for the active transport of sodium and potassium at the rates found aerobically (Whittam & Davies, 1954).

The low resistance of anoxia in tissue slices during incubation *in vitro* is a reflexion of the response of the whole animal, since adult guinea-pigs, rats and rabbits are alike in showing low tolerance to anoxia. New-born rats and rabbits, however, differ from new-born guinea-pigs in their response to anoxia, since they survive in nitrogen for appreciably longer than do new-born guinea-pigs (Fazekas, Alexander & Himwich, 1941). The survival time has recently been shown to be linearly related to the cardiac carbohydrate concentration and the maintenance of the circulation appears to be of some importance for survival (Dawes, Mott & Shelley, 1959). In view of the need also to prevent cardiac failure from an elevated plasma potassium concentration, the rather long survival in nitrogen of new-born rats and rabbits suggests the possibility that the tissues of these animals might be able to prevent a leakage of potassium into the circulation during anoxia, whilst the tissues of the new-born guinea-pig might resemble those of the adult animal in losing potassium anaerobically. This possibility has been tested with slices of kidney cortex, and the tissue sodium and potassium concentrations have been compared with the rates of respiration and of aerobic and anaerobic glycolysis. The tissue water content was also measured, as it increases in tissue slices from adult animals when

respiration is inhibited (see Robinson, 1950), and is therefore another tissue constituent whose concentration is affected by anoxia.

METHODS

Materials and procedure. Litters of rats, rabbits and guinea-pigs which had been born during the night were obtained between 9 and 10 a.m. the following morning. The animals were killed by bleeding from the carotid artery and the kidneys removed immediately. The kidneys of guinea-pigs and rabbits were bisected longitudinally and the medulla cut away with fine scissors. Slices of the cortex were made freehand with a razor blade, moistened with saline medium, by the method of Deutsch (1936), and the outermost slice was rejected. Kidneys of new-born rats weighed only about 20 mg and were too small for the poorly-defined medulla to be cut out. Two slices only were therefore cut from each rat kidney and these included the outer layer of the organ. The slices (0·3–0·4 mm thick) were placed in about 4 ml. of saline medium in a Petri dish at room temperature until they had all been made, this process taking up to 15 min. About 30–50 mg of tissue (wet wt.) were placed either in 4·0 ml. of saline medium in cups of Warburg manometers or in 3·0 ml. of saline medium in 5 ml. stoppered conical flasks. In anaerobic experiments a stick of yellow phosphorus was placed in the centre well of the manometer cups. The vessels were shaken for 1 hr in a water-bath at 37° C.

After incubation the slices were removed, rapidly blotted on hardened filter paper (Whatman No. 542) and divided into two lots, each of which was quickly weighed on a torsion balance.

Media. The two saline media contained (mM) NaCl 150, KCl 5, Na phosphate 10, pH 7·4 and glucose 10, gassed with either O_2 or N_2 for aerobic or anaerobic incubations in conical flasks; and (mM) NaCl 150, KCl 5, $NaHCO_3$ 25 and glucose 10, gassed with 5% CO_2 and 95% N_2, for anaerobic incubations in Warburg manometers.

Water content of tissue. The tissue water was taken to be equal to the loss of weight on drying the tissue during the night at 105° C.

Chemical estimations. Lactate was estimated according to the method of Barker & Summerson (1941) in the medium in which the tissue had been incubated. Sodium and potassium were estimated in extracts of the tissue by flame photometry with a lithium internal standard (Amoore, Parsons & Werkheiser, 1958). The tissue extract was obtained by dispersing the tissue in 3·0 ml. 5% (w/v) trichloroacetic acid, which was then allowed to remain at 4° C during the night. Sodium and potassium in rat kidney tissue were estimated on the dried samples on which the water content had been determined, as there was not enough tissue for analyses on separate lots of tissue. The dried sample was digested in concentrated HNO_3 as described by Whittam & Davies (1953) and the sodium and potassium contents estimated by flame photometry.

RESULTS

Cation concentrations in kidney cortex slices

Rabbit and rat. To determine whether concentration gradients of sodium and potassium could be maintained during aerobic and anaerobic incubation at 37° C, cortex slices cut from the kidneys of new-born animals were shaken in a saline medium containing glucose with a gas phase of either O_2 or N_2. The results for rabbit tissue in Table 1 are from a typical experiment of five that gave similar results. They show that the concentration of sodium was maintained at the same level during aerobic incubation, whilst

during anaerobic incubation it increased from 107 to 133 m-mole/kg wet wt. The concentration of potassium rose from 76 to 85 m-mole/kg wet wt. during aerobic incubation, but fell to 66 m-mole/kg wet wt. anaerobically. The sum of the sodium and potassium concentrations remained the same as at the start after aerobic incubation but increased 9% anaerobically. The changes in the potassium concentration were therefore approximately balanced by movements of sodium in the opposite direction. Only small changes occurred in the water content and the cation concentrations expressed in m-mole/kg dry wt. are comparable to those in m-mole/kg

TABLE 1. The sodium, potassium and water concentrations in kidney cortex slices from new-born animals

		Cation concentrations						Water concentration	
		m-mole/kg wet wt.			m-mole/kg dry wt.			ml. water/ 100 g wet wt.	ml. water/g dry wt.
Species	Conditions of incubation	Na	K	Na+K	Na	K	Na+K		
Rabbit	Before incubation	107	76	183	620	440	1060	84 ± 0·7 (3)	5·1 ± 0·3
	Aerobic	100	85	185	560	470	1030	81 ± 0·8 (5)	4·3 ± 0·2
	Anaerobic	133	66	199	780	390	1170	83 ± 1·0 (5)	4·8 ± 0·4
Rat	Before incubation	95	78	173	560	460	1020	83 ± 1·1 (5)	4·9 ± 0·4
	Aerobic	94	86	180	500	459	950	81 ± 1·2 (5)	4·4 ± 0·3
	Anaerobic	134	63	197	840	390	1230	84 ± 2·0 (5)	5·3 ± 0·8
Guinea-pig	Before incubation	116	62	178	580	310	890	80 (2)	4·0
	Aerobic	113	61	174	460	250	710	75 (2)	3·0
	Anaerobic	148	21	169	970	140	1110	85 (2)	5·6

Slices of the cortex were cut from the kidneys of a litter of new-born animals and immersed in phosphate saline medium in a Petri dish at room temperature until the slices had all been made. The tissue was then incubated at 37° C in 3·0 ml. of the phosphate saline medium, pH 7·4, containing 10 mM glucose, with a gas phase of either O_2 or N_2 for 1 hr and, after blotting and weighing the slices, the sodium, potassium and water concentrations in the tissue were estimated. The cation concentrations are mean values of duplicate measurements of a single experiment typical of at least three and the mean values for the water concentrations are shown with S.D. and the number of values in parentheses.

wet wt. Control incubations at 37° C of cortex slices cut from kidneys of the mothers of two of the litters showed a difference between the potassium concentrations aerobically and anaerobically of 43 m-mole/kg wet wt., in contrast to the mean difference (with S.D.) of 17 ± 6 found in five experiments with tissues from new-born rabbits.

Kidney cortex slices from new-born rats did not give as reproducible results as those from rabbits because of analytical difficulties due to the small amount of tissue available. The slices from fifteen new-born rats in a particularly large litter were pooled and incubated in duplicate flasks under each condition; the weight of tissue per flask was 15–20 mg and the results are shown in Table 1. They are closely similar to those obtained

with new-born rabbit tissue. The potassium concentration (in m-mole/kg wet wt.) was 86 after aerobic incubation and 63 after anaerobic incubation. The mean difference (with S.D.) in four experiments was 24 ± 7. The relatively high potassium concentration found after anaerobic incubation is in contrast with that of 18–20 m-mole/kg wet wt. which has been found after the similar incubation of kidney cortex slices from adult rats (Whittam & Robinson 1958). The sodium concentration was unchanged during aerobic incubation but increased from 95 to 134 m-mole/kg wet wt. anaerobically. The sum of the sodium and potassium concentrations consequently also remained unchanged aerobically but increased 14% anaerobically owing to the uptake of sodium. The tissue from the new-born rat therefore closely resembles, as regards the loss of potassium, the tissue from the new-born rabbit in its relative insensitivity to anaerobiosis compared with tissue slices from adult animals.

Guinea-pig. The sodium and potassium concentrations in kidney cortex slices from new-born guinea-pigs remained unchanged during aerobic incubation, but after anaerobic incubation the sodium concentration had risen from 116 to 148 m-mole/kg wet wt. and the potassium concentration had fallen from 62 to 21 (Table 1). These changes are similar to those occurring in kidney cortex slices from adult guinea-pigs, in which the potassium concentration after anaerobic incubation was 42 m-mole/kg wet wt. lower than that after aerobic incubation (Whittam & Davies, 1953). The large increase during anaerobic incubation ($67\% = 970 - 580 = 390$ m-mole/kg dry wt.) in the amount of sodium per unit weight of dry matter is made up of an uptake of sodium in exchange for potassium ($310 - 140 = 170$ m-mole/kg dry wt.) and also of a net uptake of $390 - 170 = 220$ m-mole/kg dry wt. The net uptake of sodium in kidney cortex slices from adult guinea-pigs has been shown to be accompanied by chloride (Whittam, 1956) and explains the swelling that occurs anaerobically as an entry of extracellular fluid into the cells (Leaf, 1956), and the same mechanism probably explains the present results.

Effect of metabolic inhibitors. The maintenance of the sodium and potassium concentrations found after the anaerobic incubation of kidney cortex slices of new-born rabbits and rats could be due to one of two possibilities. First, the rate of exchange of these ions might be so low that only the relatively small net changes occurred in 1 hr even though the active transport of cations might be inhibited anaerobically. Secondly, the concentrations might be maintained by active movements of ions, anaerobically as well as aerobically, at rates which would cause larger net changes in the concentrations if the active fluxes were to be inhibited by cutting off the supply of metabolic energy. To distinguish between these possibilities kidney cortex slices were incubated anaerobically in the presence of

iodoacetate, as this condition would be expected to inhibit the energy supply from both respiration and glycolysis. As an alternative way of inhibiting respiration slices were incubated aerobically with cyanide, and also, as a control, under anaerobic conditions with cyanide.

The results (Table 2) show that the potassium concentration in rabbit kidney cortex slices after anaerobic incubation with 1 mM iodoacetate was 25 m-mole/kg wet wt., indicating that iodoacetate caused a loss of (66 − 25) = 41 m-mole potassium/kg wet wt. The addition of 1 mM-KCN aerobically caused changes in the sodium and potassium concentrations to those values which were also found after anaerobic incubation, when KCN had no effect. Anoxia and the addition of KCN aerobically therefore

TABLE 2. Changes in the sodium and potassium concentrations and water content of kidney cortex slices after incubation under metabolically unfavourable conditions

	Cation concentrations (m-mole/kg wet wt.)						Water content (ml./g dry wt.)		
	Rabbit		Rat		Guinea-pig				
Species	K	Na	K	Na	K	Na	Rabbit	Rat	Guinea-pig
Before incubation	76	107	78	95	62	116	5·1	4·8	4·0
After incubation:									
Aerobic	85	100	86	94	61	113	4·3	4·3	3·0
Anaerobic	66	133	63	134	21	148	4·8	4·5	5·6
Aerobic + cyanide	62	134	63	120	—	—	5·6	5·1	—
Anaerobic + iodoacetate	25	143	35	140	19	158	6·4	6·9	5·6
Anaerobic + cyanide	60	130	62	133	—	—	5·3	5·6	—
Aerobic + cyanide + iodoacetate	29	149	—	—	—	—	7·2	—	—

Kidney cortex slices from new-born animals were incubated for 1 hr at 37° C in 3·0 ml. of phosphate saline medium containing 10 mM glucose, pH 7·4, with O_2 or N_2 in the gas phase. Potassium cyanide and Na iodoacetate were added to give a final concentration of 1 mM. The values are means of two experiments.

caused the same small leakage of tissue potassium. It seemed possible that the maintenance of a high potassium concentration aerobically with cyanide might also be due to energy from glycolysis under these conditions. Slices were therefore incubated to test whether iodoacetate, added with cyanide, under aerobic conditions, caused a greater leakage of potassium, as it did under anaerobic conditions. The results (Table 2) show that iodoacetate and cyanide added together under aerobic conditions caused a fall in potassium concentration to about 30 m-mole/kg wet wt. compared with the smaller fall to 62 with cyanide alone.

Kidney cortex slices from new-born rats resembled those from new-born rabbits. They lost the same amount of potassium (15 m-mole/kg wet wt.) aerobically, with 1 mM cyanide, as they did during anaerobic incubation,

when the addition of cyanide had no effect. Anaerobic incubation with 1 mM iodoacetate caused a leakage of 43 m-mole potassium/kg dry wt.

Cyanide (1 mM) caused a loss of potassium from new-born guinea-pig kidney cortex slices (28 m-mole/kg wet wt.) during aerobic incubation for 1 hr. In contrast to the results with rabbit and rat tissue, the addition of iodoacetate anaerobically caused only the same leakage (43 m-mole/kg wet wt.) as anaerobiosis alone (41 m-mole/kg wet wt.). The losses of potassium from tissue slices of all three species were accompanied by gains of sodium which were either approximately equal to or greater than the potassium loss (Table 2).

The results in Table 2 therefore show that an inhibitor of respiration (cyanide) caused changes in the sodium and potassium concentrations in kidney cortex slices from rabbits and rats which were the same as those produced by anaerobiosis. The addition of an inhibitor of glycolysis (iodoacetate) anaerobically caused a loss of potassium from kidney cortex slices from rabbits and rats, but not from guinea-pig tissue. The results show that metabolic energy from glycolysis was sufficient for the maintenance of high tissue potassium concentrations anaerobically in rabbit and rat kidney cortex slices but insufficient in guinea-pig kidney cortex slices.

Water content of tissue slices

Slices of kidney cortex from adult animals swell during incubation *in vitro* when the supply of energy from respiration is inhibited (see Robinson, 1950) owing to a failure of the cells to prevent the entry of sodium chloride (Whittam, 1956), with which water moves as an approximately isotonic solution (Leaf, 1956). It was therefore interesting to see whether those tissue slices from new-born animals which partially resisted anaerobiosis, as regards the regulation of their sodium and potassium concentrations, also resisted the tendency to swell. The results (Table 1) are given as the amount of water per unit dry weight of tissue and as the percentage water (g water/100 g wet wt.). They show that the water content of the rabbit tissue was 5·1 ml./g dry wt. initially, and 4·3 and 4·8 ml./g dry wt. after aerobic and anaerobic incubation; these values are not significantly different. The values for rat tissue are similar. The water content of guinea-pig kidney cortex slices, in contrast, was higher after anaerobic incubation (5·6 ml./g dry wt.) than after aerobic incubation (3·0 ml./g dry wt.), and the changes from the initial water content (4·0 ml./g dry wt.) show that during incubation the tissue lost water aerobically and gained water anaerobically. Table 2 shows the values in the presence of inhibitors. Rabbit and rat tissue swelled during anaerobic incubation with iodoacetate from 4·8–5·1 before incubation to 6·4–6·9 ml./g dry wt. The water content

of guinea-pig tissue was the same (5·6 ml./g dry wt.) after anaerobic incubation both in the presence and absence of iodoacetate.

The results show that the water content of kidney cortex slices from rabbits and rats was unchanged after aerobic or anaerobic incubation at 37° C, whilst guinea-pig kidney cortex slices swelled appreciably during anaerobic incubation. It appears that resistance to anaerobic swelling is shown by kidney cortex slices from new-born rabbits and rats but not by those of new-born guinea-pigs. In this respect, therefore, new-born rabbits and rats resemble each other and are different from adults of the species, whilst, in contrast, new-born guinea-pigs resemble adult guinea-pigs.

TABLE 3. The rates of respiration and of lactic acid production in kidney cortex slices at 37° C

Species	Conditions of incubation	Q_{O_2} (μl./mg dry wt./hr)	$Q^{N_2}_{CO_2}$ (μl. /mg dry wt./hr)	Rate of lactic acid production: μl./mg dry wt./hr	Rate of lactic acid production: m-mole/kg wet wt./hr
Rabbit	Aerobic	10 (3)	—	3·3 (2)	2·8
	Anaerobic	0	22 (3)	—	—
	Aerobic + cyanide	0	—	13·6 (3)	9·1
Rat	Aerobic	15 ± 3·7 (5)	—	3·7	3·1
	Anaerobic	0	16 (3)	—	—
	Aerobic + cyanide	0	—	9 (2)	6·6
Guinea pig	Aerobic	14	—	1	1
	Anaerobic	—	5	—	—

Slices of kidney cortex were incubated either in 4·0 ml. phosphate saline medium, pH 7·4, containing 10 mM glucose in O_2, or in 4·0 ml. bicarbonate saline medium, pH 7·4, containing 10 mM glucose gassed with 5% CO_2 and 95% N_2. The concentration of KCN was 1 mM. Lactic acid was determined in the medium after incubation. The values are means of at least two experiments; S.D. are given with the number of experiments in parentheses.

The metabolic activity of tissue slices

The resistance towards anaerobiosis by kidney cortex slices of new-born rabbits and rats, both as regards sodium and potassium concentrations and water content, suggests that they derive sufficient energy from anaerobic glycolysis to maintain a steady state close to that maintained aerobically. Measurements of the rate of oxygen consumption, of the liberation of carbon dioxide from a bicarbonate saline medium and of the lactic acid production have therefore been made to test, on the one hand, if kidney cortex slices of new-born rabbits and rats have a high rate of anaerobic metabolism and, on the other hand, if those from new-born guinea-pigs resemble adult guinea-pigs in having a low rate.

The rates of oxygen consumption by kidney cortex slices from new-born rabbits and rats at 37° C were 10 and 15 μl./mg dry wt./hr respectively,

and the rates of liberation of CO_2 anaerobically were 22 and 16 μl./mg dry wt./hr respectively (Table 3). In the rabbit tissue, the $Q_{CO_2}^{N_2}$ was therefore 2·2 times greater than the Q_{O_2}, and in the rat tissue the rates were equal. The rate of lactic acid production aerobically was about 3 m-mole/kg wet wt./hr for both the rat and rabbit tissue, and this value is equivalent to a $Q_{CO_2}^{O_2}$ (μl./mg dry wt./hr) of approximately 3·5. In contrast to the high rates of anaerobic metabolism in kidney cortex slices of the rabbit and rat, kidney cortex slices from new-born guinea-pigs were predominantly aerobic; the Q_{O_2} was 14 and the $Q_{CO_2}^{N_2}$ was 5; the rate of lactic acid production aerobically was less than 1 m-mole/kg wet wt./hr.

The O_2 uptake was completely inhibited by 1 mM-KCN, which caused an increase in aerobic lactic acid production in rabbit and rat tissue from about 3 to 9·1 and 6·6 m-mole/kg wet wt./hr respectively. Liberation of CO_2 anaerobically was 90 % inhibited by 1 mM-iodoacetate.

DISCUSSION

Cation concentrations in kidney cortex slices

Rabbits and rats are among those species whose new-born can survive anoxia for appreciably longer periods of time than new-born guinea-pigs (Fazekas *et al.* 1941), and at birth the former are less developed and are suckled for about 2 weeks whereas guinea-pigs can eat solid food. Studies on the plasma electrolytes in animals subjected to anoxia have not been made, but it may be assumed that those animals which withstand anoxia are also able to maintain a low plasma potassium concentration. If this is so, then tissues such as kidney- and brain-cortex and liver would be expected to retain their potassium during anaerobic incubation *in vitro*, in contrast to the loss which occurs from tissues from adult animals. The results show that this is substantially true for slices of kidney cortex of new-born rabbits and rats. Kidney cortex slices were found to be suitable for studies *in vitro* but the brains were too small for cutting slices. Preliminary experiments with liver slices gave results similar to kidney cortex slices but the values for the cation concentrations varied from litter to litter, probably because of variable amounts of tissue fat and glycogen.

Calculations of the cation concentrations in the intracellular fluid have not been made, as the extracellular space was not measured. Corrections for the cations in extracellular fluid would give intracellular concentrations higher for potassium and lower for sodium than those for the whole tissue. The concentrations in the whole tissue, however, show the retention of potassium at about 65 m-mole/kg wet wt. compared with a concentration of 5 mM in the medium, and the maintenance of sodium at a lower concentration (133 m-mole/kg wet wt.) than that in the medium (168 mM) by

kidney cortex slices from new-born rabbits and rats in which respiration was inhibited by incubation either anaerobically or aerobically in the presence of cyanide. The concentration gradients could not have been due to the impermeability, or very low permeability, of the cell membranes, because when glycolysis was also inhibited by the addition of iodoacetate the potassium concentration fell to about 30 m-mole/kg wet wt. The intracellular concentrations, therefore, appear to be maintained actively either by energy from respiration under aerobic conditions, when the rate of glycolysis was low, or by energy from glycolysis when respiration is inhibited by cyanide or anoxia when the rate of glycolysis was high. As the p.d. across the cell membranes is not known, it is not possible to decide whether both sodium and potassium are actively transported or sodium alone. The results show, however, that the kidney cortex of the new-born rabbit and rat can derive sufficient energy from glycolysis for the maintenance of high concentrations of potassium, and this is unlike the tissue from adult rabbits and rats which requires oxygen (Mudge, 1951; Aebi, 1953). No differences were found between the sodium and potassium concentrations in kidney cortex slices from new-born and adult guinea-pigs (Whittam & Davies, 1953), because during anaerobic incubation at 37° C potassium leaked out of the tissue in exchange for sodium, the tissue swelled and iodoacetate had no additional effect. Kidney cortex slices from the new-born rabbit and rat are, therefore, similar in their tolerance of anaerobiosis, and in this respect are different from the same tissue from new-born guinea-pigs and from adult animals of the same species.

The metabolism of kidney cortex slices

The importance of anaerobic glycolysis for the survival of animals without oxygen has been recently discussed by Dawes *et al.* (1959). The decrease in cardiac glycogen with age which they found exactly parallels the decrease in tolerance to anoxia, and supports their conclusion that the maintenance of the circulation by energy derived from glycolysis is of primary importance in the resistance to anoxia.

The present results show that glycolysis is also important for the prevention of swelling and the leakage of potassium from the cells under anaerobic conditions. Kidney cortex slices of new-born rabbits and rats liberated CO_2 from a bicarbonate saline medium at a high rate ($Q^{N_2}_{CO_2} = 22$ for rabbit and 16 for rat). The $Q^{N_2}_{CO_2}$ of 16 for kidney cortex slices of the new-born rat is only in fair agreement with the value of 11·4 found by György, Keller & Brehme (1928); comparable values for new-born rabbit and guinea-pig have not been found in the literature. The rate of lactic acid production aerobically and the $Q^{N_2}_{CO_2}$ (5) in guinea-pig kidney cortex slices were less than 30% of the corresponding values for the rabbit and rat

tissue. Since the product of both respiration and anaerobic glycolysis is adenosine triphosphoric acid (ATP) it may be inferred that a supply of ATP from either metabolic pathway may be used by the active transport systems which maintain the intracellular concentrations of sodium and potassium in kidney cortex slices of the new-born rabbit and rat.

It is instructive to compare the rates of anaerobic glycolysis ($Q^{Na}_{CO_2}$) for kidney cortex slices from new-born and adult tissues, because the values clearly demonstrate the greater capacity for glycolysis in those of the new-born rabbit and rat. Thus, the $Q^{Na}_{CO_2}$ for the tissue from adult rabbits and rats is 5 (György *et al.* 1928; Ebina, 1929) compared with respective values of 22 and 16 for the tissue of the new-born (Table 3). In contrast, the rate (6) for the tissue of the adult guinea-pig (György *et al.* 1928) is approximately equal to that for the new-born (5, Table 3).

Comparison with other tissues

Other tissues and cells which possess the ability to transport cations actively both aerobically and anaerobically are frog skin (Leaf & Renshaw, 1957), duck erythrocytes (Tosteson & Robertson, 1956), ascites tumour cells (Maizels, Remington & Truscoe, 1958) and guinea-pig seminal vesicle mucosa (Breuer & Whittam, 1957). A metabolic feature shared by tumour cells and kidney cortex slices of the new-born rabbit and rat is a rate of aerobic glycolysis higher than that of tissues such as liver and kidney cortex of adult animals (see the discussion by Warburg, 1956). Thus, per mg dry wt./hr, kidney cortex slices from the new-born rabbit consumed 10 μl. O_2 and produced 0·15 μmole lactic acid during aerobic incubation (see Table 3). If glucose is assumed to be the substrate for respiration and glycolysis, these values correspond to a glucose utilization of 0·08 μmole by respiration and also by glycolysis. The values for rat tissue are 0·12 and 0·17 respectively. The amount of glucose converted to lactic acid was, therefore, equal to that converted to CO_2 in rabbit tissue, and greater by 40 % in rat tissue. In contrast, kidney cortex slices of new-born guinea-pigs were predominantly respiratory, converting 0·10 μmole glucose to CO_2 and less than 0·04 μmole glucose to lactic acid. As regards the utilization of glucose during aerobic incubation, the production of lactic acid accounts for more catabolism than the respiratory pathway both in ascites tumour cells (Maizels *et al.* 1958) and in kidney cortex slices of new-born rabbits and rats, but not in kidney cortex slices from new-born guinea-pigs.

Another feature common to ascites cells and kidney cortex slices is a Pasteur effect (the increase in lactic acid production due to anaerobiosis) which is up to 200 % in ascites cells (Maizels *et al.* 1958), and 560 and 330 % with new-born rabbit and rat kidney cortex slices respectively. This effect

was also found with new-born guinea-pig kidney cortex slices, but since the anaerobic rate of glycolysis was only about 30 % of that in the rabbit and rat tissue the glycolytic pathway is quantitatively of less significance than respiration. The Pasteur effect, therefore, partly accounts for the high rates of anaerobic glycolysis in ascites cells, seminal vesicle mucosa and kidney cortex slices of new-born rabbits and rats.

SUMMARY

1. A study has been made of the metabolism and the sodium, potassium and water concentrations in cortex slices from the kidneys of new-born rabbits, rats and guinea-pigs during incubation under various conditions at 37° C.

2. The sodium, potassium and water concentrations in kidney cortex slices from new-born rabbits and rats were maintained during aerobic incubation for 1 hr, and the addition of 1 mM cyanide aerobically, or anaerobiosis caused only a small loss of potassium (10–15 m-mole/kg wet wt.) and no change in water content. The addition of 1 mM iodoacetate anaerobically caused a greater loss of potassium (40–50 m-mole/kg wet wt.). Kidney cortex slices from new-born guinea-pigs also maintained their cation concentrations aerobically, but they gained sodium, lost potassium and swelled during anaerobic incubation, when the addition of iodoacetate caused no further changes.

3. Kidney cortex slices from new-born guinea-pigs, therefore, resemble those from adult guinea-pigs in their sensitivity to anaerobiosis and their dependence on respiration. Slices from new-born rabbits and rats, however, maintained concentration gradients anaerobically which are compatible with the ability of these animals to withstand anoxia for longer periods than guinea-pigs.

4. The rates of liberation of CO_2 from a bicarbonate medium by kidney cortex slices from new-born rabbits and rats were 22 and 16 μl./mg dry wt./hr anaerobically, and the rate of lactic acid formation aerobically was about 3 m-mole/kg wet wt. In contrast, the respective values were 5 and less than 1 for guinea-pig tissue. The rates of oxygen consumption were also measured.

5. It is concluded that the glycolytic metabolic pathway in kidney cortex slices from new-born rabbits and rats enables active transport mechanisms to maintain sodium and potassium concentrations close to those found aerobically and to prevent swelling, whilst the predominantly respiratory pathway in the same tissue from new-born guinea-pigs accounts for its oxygen requirement for active transport.

I wish to thank Professor Sir Hans Krebs for his interest and encouragement and Dr G. S. Dawes for a helpful discussion of the manuscript.

REFERENCES

AEBI, H. H. (1953). Elektrolyt-Akkumulierung und Osmoregulation in Gewebsschnitten. *Helv. physiol. acta*, **11**, 96–121.

AMOORE, J. E., PARSONS, D. S. & WERKHEISER, W. C. (1958). A lithium internal-standard flame photometer. *Biochem. J.* **69**, 236–238.

BARKER, S. B. & SUMMERSON, W. H. (1941). The colorimetric determination of lactic acid in biological material. *J. biol. Chem.* **138**, 535–554.

BREUER, H. J. & WHITTAM, R. (1957). Ion movements in seminal vesicle mucosa. *J. Physiol.* **135**, 213–225.

DAWES, G. S., MOTT, J. C. & SHELLEY, H. J. (1959). The importance of cardiac glycogen for the maintenance of life in foetal lambs and new-born animals during anoxia. *J. Physiol.* **146**, 516–538.

DEUTSCH, W. (1936). An improvement of Warburg's method for cutting tissue slices for respiratory experiments. *J. Physiol.* **87**, 56*P*.

EBINA, T. (1929). Über den Stoffwechsel des Kaninchensarkoms. *Tohoku J. exp. Med.* **13**, 424–444.

FAZEKAS, J. F., ALEXANDER, F. A. D. & HIMWICH, H. E. (1941). Tolerance of the new-born to anoxia. *Amer. J. Physiol.* **134**, 281–287.

GYÖRGY, P., KELLER, W. & BREHME, TH. (1928). Nierenstoffwechsel und Nierenentwicklung. *Biochem. Z.* **200**, 356–366.

LEAF, A. (1956). On the mechanism of fluid exchange of tissues *in vitro*. *Biochem. J.* **62**, 241–248.

LEAF, A. & RENSHAW, A. (1957). The anaerobic active ion transport by isolated frog skin. *Biochem. J.* **65**, 90–93.

MAIZELS, M., REMINGTON, M. & TRUSCOE, R. (1958). Metabolism and sodium transfer of mouse ascites tumour cells. *J. Physiol.* **140**, 80–93.

MUDGE, G. H. (1951). Studies on potassium accumulation by rabbit kidney slices: Effect of metabolic activity. *Amer. J. Physiol.* **165**, 113–127.

ROBINSON, J. R. (1950). Osmoregulation in surviving slices from the kidneys of adult rats. *Proc. Roy. Soc.* B, **137**, 378–402.

TOSTESON, D. S. & ROBERTSON, J. S. (1956). Potassium transport in duck red cells. *J. cell. comp. Physiol.* **47**, 147–166.

WARBURG, O. (1956). On the origin of cancer cells. *Science*, **123**, 309–314.

WHITTAM, R. (1956). The permeability of kidney cortex to chloride. *J. Physiol.* **131**, 542–554.

WHITTAM, R. & DAVIES, R. E. (1953). Active transport of water, sodium, potassium and α-oxoglutarate by kidney cortex slices. *Biochem. J.* **55**, 880–888.

WHITTAM, R. & DAVIES, R. E. (1954). Energy requirements for ion transport in steady-state systems. *Nature, Lond.*, **173**, 494.

WHITTAM, R. & ROBINSON, J. R. (1958). The effect of low-sodium media on the potassium concentration of kidney-cortex slices. *Biochem. J.* **70**, 16*P*.

J. Physiol. (1960), **153**, *pp.* 370–385
With 8 text-figures
Printed in Great Britain

THE EFFECT OF SUDDEN CHANGES IN IONIC CONCENTRATIONS ON THE MEMBRANE POTENTIAL OF SINGLE MUSCLE FIBRES

By A. L. HODGKIN and P. HOROWICZ*
From the Physiological Laboratory, University of Cambridge

(*Received* 20 *May* 1960)

In an earlier paper on single muscle fibres (Hodgkin & Horowicz, 1959), we showed that the displacements of membrane potential produced by changes in the external ionic concentration were quantitatively consistent with the idea that the membrane potential is determined by the concentration ratios of the potassium and chloride ions. Without making further measurements it might be supposed that the rate of change of membrane potential in response to a sudden alteration of $[K]_o$ or $[Cl]_o$ would also be of a relatively simple kind. If ions in the external solution could reach the membrane without appreciable delay, the rate of change of membrane potential should be determined by the membrane time constant, which is normally about 30 msec (Fatt & Katz, 1951). If there were a straightforward diffusion delay it should be possible to measure the delay in one record and to predict the shape of all other records. The results described here show that the actual situation is more complicated, the main findings being that $[Cl]_o$ affects the membrane potential more rapidly than $[K]_o$ and that the depolarization associated with a rise of $[K]_o$ is quicker than the repolarization associated with a fall. A partial explanation is that the sites which are sensitive to potassium ions are less accessible than those which are sensitive to chloride and that appreciable quantities of potassium can be retained in a special region of the fibre. This suggestion accounts for a number of puzzling observations, but something further is needed to explain why the effect of a rise in $[K]_o$ is so much more rapid than that of a fall.

In the present paper the words rapid and slow have a different meaning from those used in our previous article (Hodgkin & Horowicz, 1959). In describing the earlier results, any change taking place in less than a minute was regarded as 'rapid' and the word 'slow' was reserved for the drifts in membrane potential, lasting minutes or hours, that are associated

* Present address: Department of Physiology, Washington University, St Louis, Missouri, U.S.A.

with movements of KCl into or out of the fibre. Here, a 'rapid' change is one that takes place in a fraction of a second and a 'slow' change is one occurring in seconds or tens of seconds.

METHODS

The apparatus and general procedure were identical with those described in the previous paper (Hodgkin & Horowicz, 1959). In most cases, membrane potentials were recorded between an internal micro-electrode, filled with 3 M-KCl, and a similar electrode in the external solution. At the end of each experiment tests of the kind described in the previous article were made to check that the electrodes were not influenced by junction potentials. One of these tests, which consisted in flushing the cell with a new solution and recording the change in p.d. between the external micro-electrode and an external agar-Ringer electrode, provided information about the rate of change of concentration in the cell.

Since the experiments dealt with sudden changes in concentration it was desirable to introduce the new solution as rapidly as possible. The usual procedure was to flush 2–5 ml. of solution through the cell in a period of 1–2 sec; the rate of flow varied between 1 and 3 ml./sec. The total volume in the cell was about 0·3 ml. and the volume between the point of impalement and the tap was about 0·1 ml. The cross-sectional area of the cell was 0·09 cm^2, so a flow of 2 ml./sec corresponded to a mean velocity of about 20 cm/sec. It is difficult to give a figure for the rate of change of concentration in the immediate vicinity of the fibre. When Ringer's fluid was replaced with a solution such as sodium sulphate, a junction potential developed between the external micro-electrode, which was filled with 3 M-KCl, and the agar-Ringer electrode. This potential difference, which should not reach its final value until the solution between the two electrodes had been fully changed, was 75 % complete in 0·1 sec at flows of 2 ml./sec. However, it is doubtful whether this figure is a true indication of the rate of change in concentration near the fibre, for an unstirred layer close to the fibre might retard the change in concentration. The best evidence that the solution near the fibre was being changed rapidly is provided by the observation that changes in membrane potential produced by alterations of chloride concentration at flows of 2 ml./sec were 50 % complete in 0·2 sec.

The main difficulty with the method was that low rates of flow did not change the solution rapidly enough and that high rates tended to jerk the micro-electrode and to give a momentary depolarization of a few millivolts. These artifacts could be recognized as such, since they occurred when the solution already in the cell was flushed through for a second time.

Solutions. The solutions used were similar to those described in a previous paper (Hodgkin & Horowicz, 1959, Table 1). Chloride was replaced by SO_4 or mixtures of SO_4 and sucrose; $CaSO_4$ was added to maintain the level of ionized calcium. Since $[K]_o$ and $[Cl]_o$ were the important variables the solutions will be specified by the concentrations (in mM) of these ions. Thus Ringer's fluid with 2·5 mM-K and 120 mM-Cl is designated 2·5-K 120-Cl.

A control experiment with a modified method. In the standard method the fibre rested on a glass pedestal which had been lightly coated with petroleum jelly. One side of the fibre was in contact with a layer of grease over a length of about 300 μ. Although the area of membrane not directly exposed to the solution was small, it seemed just possible that retention of potassium between the grease and the fibre might account for the slowness of repolarization following a reduction of potassium concentration. Two experiments were therefore carried out with fibres which were suspended freely in the solution. Chloride-free solutions containing sulphate were employed, and the change in potassium concentration was from 80 to 2·5 mM-K. With no pedestal, the micro-electrode was jerked out of the fibre during the change in solution but usually reimpaled the fibre immediately afterwards. Although the records obtained by this method were rather irregular, they showed that

repolarization after a reduction of $[K]_o$ was as slow in these experiments as in those with a pedestal. This indicates that retention of potassium between the grease and the fibre was not the explanation of the slow response to a reduction of $[K]_o$.

RESULTS

Changes in membrane potential produced by alterations of $[Cl]_o$

Figure 1 illustrates the effect of changing $[Cl]_o$ at constant $[K]_o$ on the membrane potential of fibres equilibrated in various solutions. In all cases the change was 50 % complete in less than 0·3 sec but there was often a slow drift to the final level. This was most conspicuous when fibres loaded with chloride were suddenly exposed to a solution containing little chloride— for example record *D*. The duration of the slow component was of the same order as the repolarization caused by a fall in $[K]_o$ and it seems

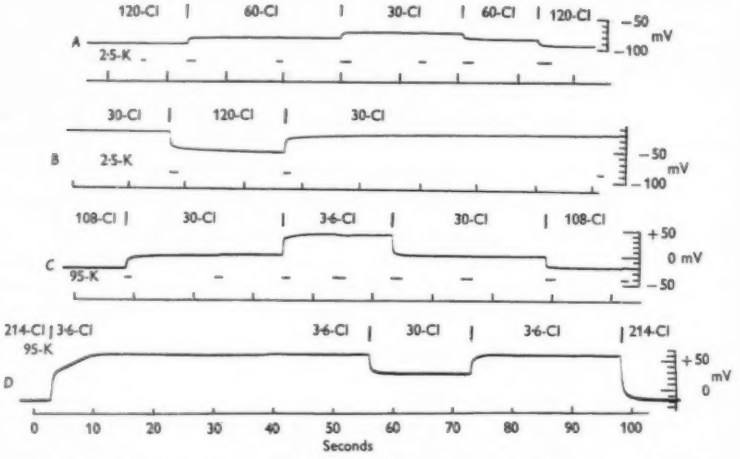


Fig. 1. Rapid effect on internal potential of changing $[Cl]_o$ at constant $[K]_o$. The concentrations of K and Cl, in mM, are shown on the records. The fibres had been equilibrated in the following solutions: *A*, 2·5-K 120-Cl; *B*, 100-K 30-Cl; *C*, 95-K 108-Cl; *D*, 95-K 214-Cl. In this and subsequent figures the solutions used in any one experiment always had the same tonicity. Chloride was replaced with sulphate, or sulphate and sucrose, using solutions of the type described in Hodgkin & Horowicz (1959, Table 1); for example, in *A* 2·5-K 120-Cl is Ringer's fluid (solution *A* of that Table) and 2·5-K 30-Cl is solution *G* of the same Table. Fibre reference numbers and diameters were as follows: *A*, 29, 111 μ; *B*, 27, 147 μ; *C*, 19, 92 μ; *D*, 17, 133 μ. Rates of flow were 1–3 ml./sec and temperatures 19–23° C. The horizontal lines below records *A*, *B* and *C* mark the periods when solution was flowing through the cell; vertical lines above the record mark changes in composition; note that there were some control flushes with the solution already in the cell. The horizontal lines marking flushes were taken from the transducer output— see Fig. 2. The flushes in *D* were about 10 sec in duration.

possible that whatever slows the response to $[K]_o$ may also have some effect on the response to $[Cl]_o$. However, it is difficult to be sure that the slow component seen with $[Cl]_o$ as the variable is not an artifact. Thus a record such as *D* can be explained by assuming that 80–90 % of the solution near the fibre was changed quickly and that it took a few seconds to wash out the remaining 10–20 %.

Comparison of effects on membrane potential of changing $[K]_o$ *and* $[Cl]_o$

Figure 2 illustrates the difference between the effect of changing $[K]_o$ at constant $[Cl]_o$ (record *A*) with that of changing $[Cl]_o$ at constant $[K]_o$ (records *B* and *C*). The experiment shows that although the effect of a rise in $[K]_o$ occurs at much the same rate as that found with $[Cl]_o$ the effect of a fall in $[K]_o$ is much slower. Thus the half-time of the repolarization in record *A* is 2·3 sec as against 0·2 sec for the half-times in records *B* and *C*.

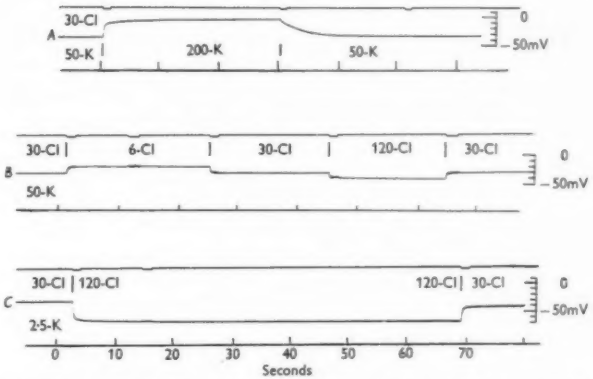


Fig. 2. Comparison of effect of changing $[K]_o$ at constant $[Cl]_o$, record *A*, with effect of changing $[Cl]_o$ at constant $[K]_o$, record *B* (50-K) and *C* (2·5-K). Chloride was replaced with SO_4 and K with Na. The fibre had been equilibrated in 50-K 30-Cl and was transferred to 2·5-K 30-Cl just before record *C*. The lower line is the internal potential and the upper line is the transducer output. Fibre 28, diameter 134 μ, temperature 22° C.

The asymmetry between the on- and off-effects of K is not explained by non-linearity in the relation between membrane potential and potassium concentration. If the potassium concentration at the membrane rose and fell in a symmetrical manner the curves for the rise and fall of $[K]_o$ should cross at a potassium concentration of $\frac{1}{2}(50+200) = 125$ mM. From other measurements made on the same fibre the potential at this concentration is estimated as -15 mV. Now the time to depolarize to -15 mV is 0·3 sec,

whereas the time to repolarize to -15 mV is 1·3 sec. The conclusion is that the 'on-effect' of raising $[K]_o$ occurs more rapidly than the 'off-effect' of reducing $[K]_o$.

The asymmetrical response to $[K]_o$

Variation of $[K]_o$ *in the absence of chloride.* The difference between the on- and off-effects of raising $[K]_o$ is well illustrated by Fig. 3, record *A*, which shows the effect of the changes 2·5 → 15 → 2·5-K in the absence of chloride. The half-time of the on-effect is about 0·3 and for the off-effect 3·2 sec. The shape of the record suggests that the depolarization may take place in two stages, i.e. in (i) a rapid step whose rate is probably limited by the time taken to change solutions, and (ii) a slower creep with a time constant of a few seconds. When $[K]_o$ was reduced the rapid step was small and almost the whole of the repolarization occurred slowly.

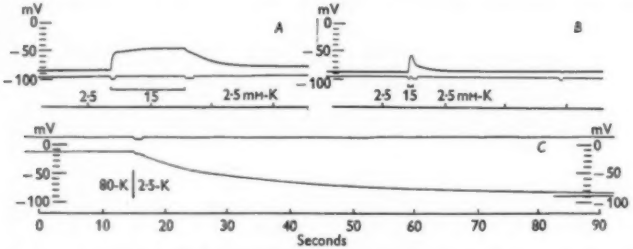


Fig. 3. Effect of varying $[K]_o$ in the absence of $[Cl]_o$. The transducer output is below the potential in *A* and *B* and above it in *C*. The records were taken in the order *C*, *B*, *A*. The fibre had been in 80-K 0-Cl for 3 min before the beginning of record *C* and had previously been in 2·5-K 0-Cl for 2 hr. The solutions contained sulphate and sucrose and had the same tonicity and ionic strength as Ringer's fluid. The 80-K 0-Cl solution was identical with *E*, Table 1 (Hodgkin & Horowicz, 1959). The other two solutions were intermediate between *D* and *E* of that table. Fibre 24, 119 μ, 18° C; flow 2 ml./sec.

This was not true if the potassium concentration was raised for only a brief time. In Fig. 3*B* the potassium concentration was raised for 0·7 sec; under these conditions the rapid step was larger and the amplitude of the slow component was reduced. Further information about the effect of varying the duration of the exposure to raised $[K]_o$ on the rate of repolarization is given in Fig. 4. These records were obtained by switching back and forth between 2·5 and 10-K for different periods.

When high potassium concentrations were employed the rate of repolarization became very slow. Figure 3*C*, which was obtained on the same fibre as Fig. 3*A* and *B*, illustrates the effect of reducing $[K]_o$ from 80 to 2·5 mM. The fibre repolarized from -17 to -95 mV but the half-time

was 12 sec and repolarization was not complete until about 100 sec after the change.

Figure 5 illustrates the way in which the membrane potential approached its equilibrium value after a number of different changes in potassium concentration. The effect of the change 2·5 → 80-K was difficult to study, because the fibre developed maximal tension for a few seconds and was damaged irreversibly if the electrode was left in position during the transient contraction. However, if the fibre was kept for most of the time in 80-K, and 2·5-K was applied for short periods, there was insufficient

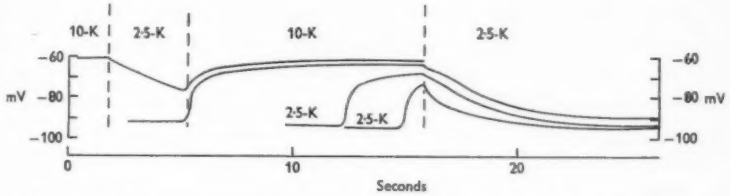


Fig. 4. Effect on internal potential of switching between 10-K 0-Cl and 2·5-K 0-Cl for different periods, using isotonic sulphate solutions of same type as in Fig. 3. Fibre 23, 130 μ, 19° C. The flow was 2 ml./sec and the duration of the flushes about 1 sec.

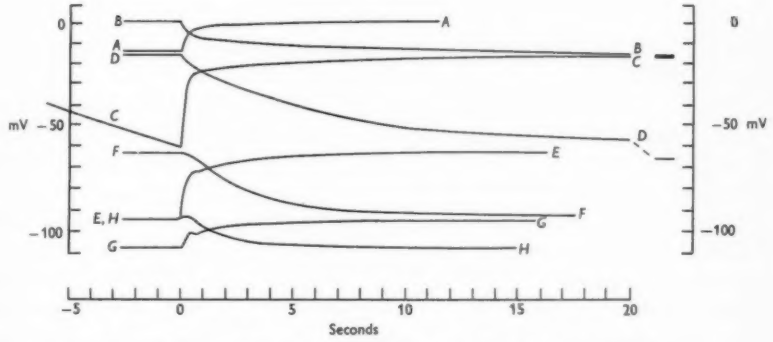


Fig. 5. Tracings showing on- and off-effects of K on membrane potential

A	80 → 190-K	*E*	2·5 → 10-K
B	190 → 80-K	*F*	10 → 2·5-K
C	2·5 → 80-K	*G*	1 → 2·5-K
D	80 → 10-K	*H*	2·5 → 1-K

Isotonic, chloride-free, sulphate solutions of the type used previously (Hodgkin & Horowicz, 1959, Table 1, solutions *D*, *E*, *H*). Fibre 23, 130 μ, 19° C. The flow rate was 2 ml./sec and the duration of the flushes were: *A* and *B*, 0·6 sec; *C*, 1·4 sec; *D*, 0·7 sec; *E*, 0·7 sec; *G* and *H*, 0·5 sec. A small flush artifact can be seen in records *E*, *G* and *H*.

time to prime the contractile mechanism and the fibre did not develop tension when 80-K was reapplied. Record *C* in Fig. 5 was obtained in this way.

Variation of $[K]_o$ *in the presence of* $[Cl]_o$. In the experiments considered so far the fibres were in solutions which contained both sodium and sulphate ions. It is clearly important to know whether similar phenomena can be observed in media which are free from these ions. Records *A–F* in Fig. 6 were obtained by varying $[K]_o$ at constant $[Cl]_o$ with no sulphate in the solution and in *A–D* sodium ions were replaced with choline. Since chloride ions entered the fibre when it was depolarized the potential did not return to its original value when 2·5-K was restored. However, with the relatively short exposures used in *A–E* this effect was small and did not obscure the marked asymmetry in the effect of $[K]_o$. The rate of repolarization was greater than in the absence of chloride (see p. 383), but in other respects the form of the curves was similar to that observed in sulphate. Curves *G* and *H*, which are included for the sake of completeness, illustrate the repolarization in Ringer's fluid after treatment with 190-K 3·6-Cl. Curve *I* has been replotted from the only experiment in which we were able to record the potential during a maximal contraction.

Correlation of the time constant of repolarization with fibre diameter

A comparison of records *B* and *D* in Fig. 6 suggests that the rate of repolarization might be slower in fibres of large diameter. Since the half-time of repolarization varied greatly with the degree of depolarization and with the solution used to restore the resting potential, it was essential in comparing different fibres to use records obtained with the same change in solution. Table 1 gives the results obtained in four different types of experiment with fibres varying in diameter between 62 and 160 μ. In Group II, where the range of fibre diameters is small, and III, where there are only three experiments, there is no significant correlation between diameter and time constant. In I the range was from 74·5 to 148 μ and there is a significant correlation with $r = 0{\cdot}71$ and $0{\cdot}02 < P < 0{\cdot}05$. In IV the range was from 62 to 122 μ and the correlation was significant with $r = 0{\cdot}93$ and $0{\cdot}02 < P < 0{\cdot}05$. If the four groups were normalized and treated as a whole, the correlation was again significant with $r = 0{\cdot}76$ and $P < 0{\cdot}001$. Although the evidence rests rather heavily on a few fibres it seems reasonable to conclude that repolarization is slower in large fibres than in small.

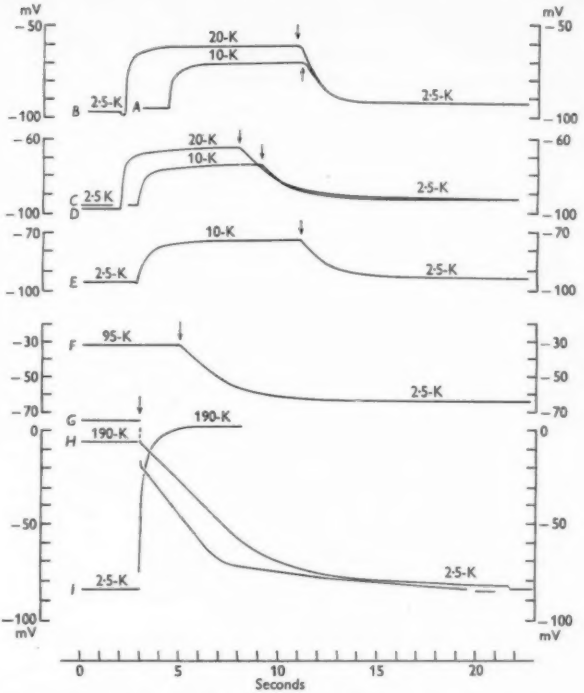


Fig. 6. Repolarization in 2·5-K 120-Cl (or 214-Cl) after depolarization with 10–190-K. The potassium concentrations (mM) are shown on the records and other experimental details are given below.

Curve	Fibre reference	Fibre diameter (μ)	Notes on solutions
A and *B*	7	74·5	120–Cl throughout (choline)
C and *D*	6	138	120-Cl throughout (choline)
E	25	119	120-Cl throughout (Na)
F	21	132	214-Cl throughout (Na)
G	3	83	190-K 3·6-Cl → 2·5-K 120-Cl (Na)
H	2	62	190-K 3·6-Cl → 2·5-K 120-Cl (Na)
I	2	62	2·5-K 120-Cl (Na) → 190-K 3·6-Cl

Curves *B* and *D* have been shifted downwards 3 and 6 mV respectively in order to superpose the end of the records. In *F* the fibre had been in high K for 1·7 min, in *G* for 11 min and in *H* for 3 min. Curves *E* and *F* were recorded with a 3 M-KCl-filled micro-electrode in the external circuit; the remainder with an agar-Ringer electrode externally. Corrections for junction potentials (a 12 mV step) have been made in *G–I*, but are unnecessary (< 1 mV) in the other cases. Curves *H* and *I* have been rescaled, the others are tracings. The fibre did not contract in any case except *I*, where it developed maximum tension for 1·5 sec and then relaxed with a time constant of about 1 sec. Temperature 18–21° C.

TABLE 1. Half-times of repolarization ($t_{\frac{1}{2}}$) and fibre diameter

Fibre reference	Temperature (°C)	Flow	Time in 10-K (min)	Diameter (μ)	$t_{\frac{1}{2}}$ (sec)
		Group I, 10-K → 2·5-K; 120-Cl, choline			
7	18	m	0·12	74·5	0·70
8	20	s	0·10	93	0·75
11	19	s	0·4	96	1·22
13	20	s	0·30	102	1·24
10	19	q	0·20	126	1·34
9	19	q	0·10	134	1·09
6	21	m	0·10	138	1·07
12	20	s	1·0	148	1·85
	Mean			114	1·16
		Group II, 10-K → 2·5-K; 120-Cl, Na			
4	21	—	0·19	75	0·8
14	19	s	0·30	85	1·1
15	19	s	0·23	87	1·0
16	20	q	0·2	118	1·0
25	20	—	0·13	119	1·1
	Mean			97	1·0
		Group III, 10-K → 2·5-K; 0-Cl, Na			
23	19	m	0·4	130	2·3
22	19	m	0·2	137	3·3
26	19	m	0·3	160	3·6
	Mean			142	3·1
		Group IV, 190-K 3·6-Cl → 2·5-K 120-Cl			
			Time in 190-K (min)		
2	21	—	2·3	62	2·6
4	21	—	7	75	4·1
3	21	—	11	83	3 7
5	—	—	4·5	83	5·5
1	20	—	*ca.* 10	122	7·4
	Mean			85	4·7

Under flow, m (medium) means a flow of about 2 ml./sec, q (quick) about 3 ml./sec and s (slow) about 1 ml./sec. In Group IV the potentials were recorded with an agar-Ringer electrode and $t_{\frac{1}{2}}$ has been measured without correcting for the junction potential.

Transient repolarizations produced by variations of $[K]_o$ *in muscles loaded with chloride*

Figure 7, record *A*, illustrates the effect of changing from 95-K 30-Cl to 2·5-K 214-Cl in a fibre which had been equilibrated for several hours in the former solution. The change in membrane potential, which was almost exactly that calculated for a chloride electrode, took place in two stages. There was first an initial rapid step of 10 mV and later a slower repolarization of 40 mV lasting about 5 sec. In other experiments it was shown that these two components could be separated by making the change in two steps, using the sequence

95-K 30-Cl → 95-K 214-Cl

95-K 214-Cl → 2·5-K 214-Cl.

The increase in chloride then gave the rapid 10 mV step and the decrease in potassium gave the slower 40 mV repolarization. The reason why increasing chloride gave only a small step is that the fibre was kept depolarized by the high external concentration of potassium. It was not until the potassium concentration was reduced that external chloride ions could establish their equilibrium potential.

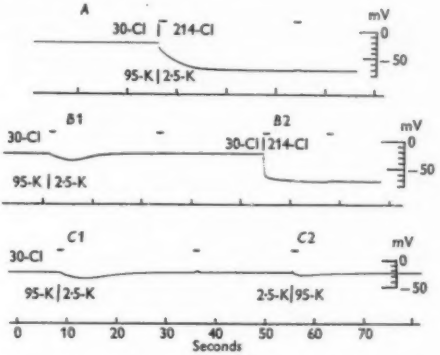


Fig. 7. Effect of varying $[K]_o$ and $[Cl]_o$ simultaneously, *A*, or separately *B* 1, *B* 2, *C* 1 and *C* 2. Fibre 20, 75 μ, 20° C, equilibrated in 95-K 30-Cl (83 mM-SO_4 and 147 mM-sucrose). The tonicity of all solutions was 1·75 times Ringer's fluid.

In the experiment of Fig. 7 we were not primarily concerned with the changes which have just been described but with the alternative sequence

95-K 30-Cl → 2·5-K 30-Cl (record *B* 1)

2·5-K 30-Cl → 2·5-K 214-Cl (record *B* 2).

The first change, record *B* 1, gave a transient repolarization, but after 15 sec the potential in 2·5-K 30-Cl was the same as in 95-K 30-Cl. The reason is that in 2·5-K there is a large outward driving force on the potassium ions and that the potassium permeability is reduced to a low value. At intermediate potassium concentrations, for instance, 25 mM, the potassium permeability is higher than in 2·5 mM-K and the resting potential is larger (*V* more negative) than in either 95 mM-K or 2·5 mM-K (Hodgkin & Horowicz, 1959, p. 146). Setting aside record *B* 1 for the moment, records *A* and *B* 2 can be reconciled by assuming that chloride ions can reach certain parts of the membrane more rapidly than potassium ions can leave the sites at which they act. Chloride ions did not restore the membrane potential at once in *A* because 'external' potassium ions were still present near the K-sensitive sites. In record *B* 2 potassium ions had already been washed out, so chloride ions exerted their effect in a rapid manner.

Having dealt with this point the transient repolarization in $B1$ (Fig. 7) will now be considered. Records $C2$ and $C1$ show that there was a transient repolarization when $[K]_o$ was suddenly increased from 2·5 to 95 as well as when it was suddenly reduced from 95 to 2·5. A possible explanation of both transients is that the resting potential was higher (V more negative) at potassium concentrations between 2·5 and 95 than it was in either solutions and that as the local concentration passed through the intermediate region it gave a temporary repolarization.

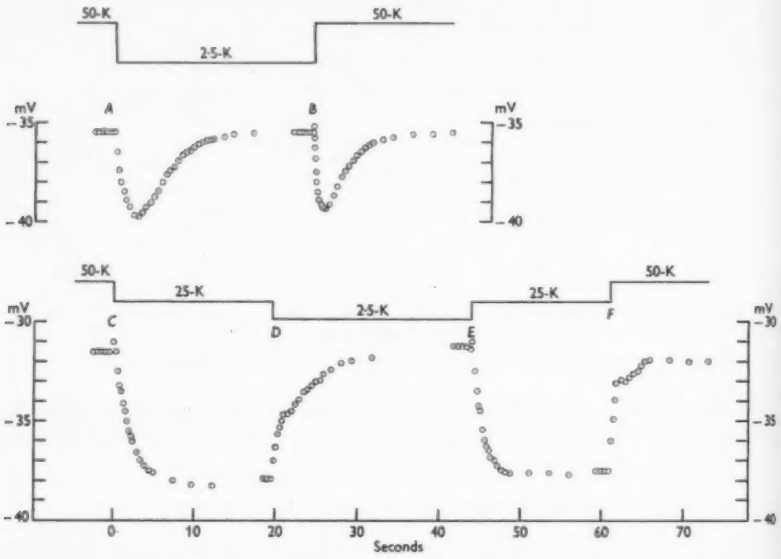


Fig. 8. Effect of changing $[K]_o$ at constant $[Cl]_o$ (30 mM) on the internal potential of a fibre equilibrated in 50-K 30-Cl. C–F were obtained 80 min after A and B. The transients in A–B would probably have been smaller if they had been measured at the same time as C–F. From the same experiment as Fig. 12 of Hodgkin & Horowicz (1959). The points were obtained by measuring greatly magnified projections of the original records. Fibre 28, 134 μ, 22° C.

Figure 8 supports this explanation. This fibre had been equilibrated in 50-K 30-Cl and in A and B the effects of the change 50 → 2·5-K or 2·5 → 50-K were recorded. As in the previous example, there were transient repolarizations after both changes but no permanent displacement of membrane potential. Curves C–F, which give the effect of the changes 50 → 25 → 2·5 → 25 → 50-K, show that in 25-K the resting potential was higher than in the other two solutions. Since the steady rise in resting potential at 25-K was greater than the transient repolarizations in the

changes 50-K → 2·5-K or 2·5 → 50-K, it is plainly reasonable to attribute the transients to the presence in a local region of concentrations intermediate between those in the two solutions. The maximum change in potential during the transient should not be larger than the steady rise at the optimum potassium concentration, but it might well be smaller since it is unlikely that potassium would be lost at the same rate from all sites.

An interesting point illustrated by Fig. 8 is that the depolarization in *D* is slower than the repolarization in *E*. This is the reverse of the usual situation, in which depolarizations are quicker than repolarizations, but the result is in keeping with the conclusion that the local potassium concentration rises more rapidly than it falls. In other words the asymmetry between the on- and off-effects of potassium depend more on the direction of the change in potassium concentration than on the direction in which the membrane potential is altering. The conclusion fits with the observation that the transient repolarization associated with the change 2·5 → 50-K is quicker than that associated with the converse change.

Quantity of K^+ retained near membrane

A partial explanation of the slowness of the response to a reduction of $[K]_o$ is that appreciable quantities of external K^+ are retained in a special region just outside the K-selective parts of the membrane. A minimum value for the quantity of K^+ in this region can be calculated by a method which will be illustrated by Fig. 6, record *F*. In this experiment a fibre which had been in 95-K 214-Cl for 104 sec was suddenly exposed to 2·5-K 214-Cl. The fibre repolarized from -32 to -64 mV with a time constant of about 2 sec. From other measurements it is known that in an experiment of this kind a fibre repolarizes to the chloride equilibrium potential, V_{Cl} (Hodgkin & Horowicz, 1959). It has also been shown that chloride movements agree with the constant field equation and that P_{Cl} is about 4×10^{-6} cm/sec. (The value found in this particular experiment was $3{\cdot}8 \times 10^{-6}$ cm/sec.) Since V is known from the record, it is possible to calculate the rate at which Cl^- enters the fibre as a function of time. The equation used was

$$M_{Cl} = P_{Cl}[Cl]_o \frac{VF}{RT} \frac{(1 - e^{(V_{Cl}-V)F/RT})}{1 - e^{-VF/RT}}, \qquad (1)$$

where M_{Cl} is the flow of chloride into the fibre in pmole/cm²sec, V is the internal potential, V_{Cl} is the chloride equilibrium potential which is taken from the final level at the end of the record, P_{Cl} is the chloride permeability (4×10^{-6} cm/sec) and the other symbols have their usual significance.

The total quantity of chloride which enters the fibre during the period after the change in solution was then obtained by numerical integration as

$$Q_{Cl} = \int_{t=0}^{\text{final}} M_{Cl}\, dt. \qquad (2)$$

In the experiment of Fig. 6, record F, Q_{Cl} came to 617 pmole/cm². Of this at most 4 pmole/cm² were required to charge the membrane capacity; the remainder was presumably balanced by an inward movement of K^+ from the special region near the membrane. There should therefore have been at least 613 pmole/cm² of K^+ in the special region. This quantity is a minimum because some K^+ should also diffuse from the special region into the external solution. If the initial and final concentrations of K^+ in the special region are assumed to be the same as those in the external solution it is possible to calculate the minimum volume of the region as

$$Q_K/\{[K]_{initial} - [K]_{final}\}.$$

For the experiment which has just been considered, the minimum volume of the special region was found to be 0·002 of the fibre volume. Values obtained in eleven other experiments varied between 0·0018 and 0·0059.

TABLE 2. Calculated entry of chloride during period while fibre is repolarizing

1 Fibre reference	2 Diameter (μ)	3 Time in solution 1 (min)	4 Solution 1 $[K]_1$ (mM)	4 Solution 1 $[Cl]_1$ (mM)	5 Solution 2 $[K]_2$ (mM)	5 Solution 2 $[Cl]_2$ (mM)	6 V_1 (mV)	6 V_2 (mV)	7 $t_{\frac{1}{2}}$ (sec)	8 $Q_{Cl}(\doteqdot Q_K)$ calculated (pmole/cm²)	9 Fraction of fibre volume
6	138	0·10	10	120	2·5	120	− 75	− 94	1·1	50	0·0020
6	138	0·10	20	120	2·5	120	− 59	− 86	1·6	133	0·0022
6	138	0·10	30	120	2·5	120	− 47·5	− 76	1·5	173	0·0018
7	74·5	0·12	10	120	2·5	120	− 70·5	− 92	0·7	38	0·0027
7	74·5	0·15	20	120	2·5	120	− 58	− 89	0·7	73	0·0022
15	87	0·23	10	120	2·5	120	− 72·5	− 93	1·0	52	0·0032
25	119	0·13	10	120	2·5	120	− 72·5	− 93	1·1	65	0·0029
21	132	1·7	95	214	2·5	214	− 32	− 64	1·7	617	0·0029
20	75	215*	95	30	2·5	214	− 23·5	− 71·5	2·0	1000	0·0058
18	135	240*	95	3·6	2·5	214	− 21	− 84	4·3	1856	0·0059
2	62	2·3	190	3·6	2·5	120	− 5	− 86	2·6	885	0·0030
3	83	11	190	3·6	2·5	120	+ 6	− 83	3·7	1430	0·0057

* In these cases other solutions besides 1 were employed for some of the time but the fibre was depolarized for the period shown. Q_{Cl} in column 8 was calculated by eqns. 1 and 2, and is the quantity of Cl per cm² of membrane which should enter the fibre while it is repolarizing. It is assumed that Q_K, the quantity of K accompanying Cl, is equal to Q_{Cl}. Column 9 gives the volume required to contain the potassium at the same concentration as in the external fluid.

It will be seen from Table 2 that the largest volumes were observed in fibres which had been kept for a relatively long time in solutions of high potassium concentration. This is probably not a coincidence since there were other indications that the repolarization became unusually slow after prolonged immersion in a low-Cl high-K solution.

On the hypothesis advanced it is possible to estimate how far the minimum volume calculated for the special region is likely to be in error. When the fibre repolarizes in a chloride-free sulphate solution there should be no movement of potassium into the fibre and the rate of repolarization should be determined by the rate at which potassium ions diffuse from the

special region into the external fluid. Repolarization in sulphate ought therefore to be slower than in a medium containing chloride. If the rate were much lower, the estimate of the minimum volume should not be seriously in error. On the other hand if the two rates were nearly equal the actual volume would clearly be much greater than the minimum obtained by calculating the quantity of K^+ which moves inwards with Cl^-. Table 1 indicates that the rate of repolarization in sulphate was 2–3 times slower than in chloride and this result is supported by an experiment in which the following changes were compared on the same fibre:

80-K 0-Cl → 2·5-K 0-Cl

80-K 0-Cl → 2·5-K 120-Cl.

In the first case the repolarization was half complete in 10 sec and in the second in about 5 sec. From the records it was calculated that the initial loss of potassium ions from the special region might occur 2–3 times faster in presence of chloride. The tentative conclusion is that the actual volume of the special region might be 50–100 % greater than the minimum values in Table 2.

DISCUSSION

The experiments described in this paper bring out a number of puzzling phenomena for which there is at present no clear explanation. The main facts established are, first that chloride ions affect the membrane potential of a muscle fibre more rapidly than potassium ions, and secondly that the repolarization resulting from a fall in $[K]_o$ is slower than the depolarization resulting from a rise in $[K]_o$. In order to explain the slowness of repolarization it seems necessary to suppose either that some charged particle besides K^+ and Cl^- can have transient effects on the membrane potential or that appreciable quantities of K^+ are retained in a special region whose volume is greater than 1/500 of that of the fibre (Table 2).

In attempting to understand the new observations one naturally turns to the evidence about the system of tubules and vesicles which have been seen in electron micrographs of skeletal muscle (Bennett & Porter, 1953; Edwards & Ruska, 1955; Robertson, 1956; Porter & Palade, 1957). There are also the important experiments of Huxley & Taylor (1958) which show that highly localized contractions can be obtained if certain sensitive spots on the surface of a muscle fibre are depolarized electrically. In frog muscle the sensitive spots are at the level of the Z lines and the contraction involves shortening of the I bands in either side of the Z line. The contraction may spread inwards for as much as 10 μ but does not involve adjacent sarcomeres. Huxley & Taylor suggested that their results might be explained if some of the tubules were electrically continuous with the external fluid and if activation of the mechanical system were brought

about by an electrical spread of depolarization along the tubule. As Huxley (1959) has pointed out, the simplest form of the hypothesis, in which the tubules are open at the external surface, is not supported by present evidence from electron microscopy. However, if the tubules were closed by an unselective membrane of high permeability there should be electrical continuity between the external fluid and the lumen of the tubule. If there is electrical continuity, external potassium ions should diffuse into the tubules and they might alter the internal potential of the muscle fibre by changing the potential difference across the walls of the tubule. The action of chloride might be more rapid than that of potassium if Cl^- exerted its effect on the outer surface of the fibre and K^+ had the greater influence on the tubular membrane. Since the distance which K^+ diffuses should increase with fibre diameter the hypothesis explains why the time constant of repolarization is longer in large fibres than in small. It also accounts for the observation that after a short exposure to high $[K]_o$, in which K^+ should not diffuse far into the tubule, recovery of the normal resting potential is quicker than after a longer exposure. A disadvantage of the hypothesis is that it does not explain why changes in potential produced by a rise in $[K]_o$ were more rapid than those associated with a fall. The asymmetry may be connected with the curious rectifying properties of the potassium channel in muscle (Katz, 1949), but it is difficult to go further with this idea until more is known about the rectification.

SUMMARY

1. In single muscle fibres the change in membrane potential produced by a sudden fall in potassium concentration was found to be slower than the change produced by a rise in potassium concentration or by an alteration of chloride concentration in either direction.

2. The asymmetry between the on- and off-effects of K^+, which was observed both in presence and absence of Cl^-, was too great to be explained by the logarithmic relation between potential and concentration.

3. The repolarization resulting from a fall in $[K]_o$ was slower in large fibres than in small.

4. A partial explanation is that potassium ions can be retained for a short time in a special region whose volume is tentatively estimated as 1/500 to 1/200 of the fibre.

REFERENCES

Bennett, H. S. & Porter, K. R. (1953). An electron microscope study of sectioned breast muscle of the domestic fowl. *Amer. J. Anat.* **93**, 61–105.

Edwards, G. A. & Ruska, H. (1955). The function and metabolism of certain insect muscles in relation to their structures. *Quart. J. micr. Sci.* **96**, 151–159.

Fatt, P. & Katz, B. (1951). An analysis of the end-plate potential recorded with an intracellular electrode. *J. Physiol.* **115**, 320–370.

HODGKIN, A. L. & HOROWICZ, P. (1959). The influence of potassium and chloride ions on the membrane potential of single muscle fibres. *J. Physiol.* **148**, 127–160.

HUXLEY, A. F. (1959). Local activation of muscle. *Ann. N.Y. Acad. Sci.* **81**, 446–452.

HUXLEY, A. F. & TAYLOR, R. E. (1958). Local activation of striated muscle fibres. *J. Physiol.* **144**, 426–441.

KATZ, B. (1949). Les constantes électriques de la membrane du muscle. *Arch. Sci. physiol.* **3**, 285–299.

PORTER, K. R. & PALADE, G. E. (1957). Studies on the endoplasmic reticulum. III. Its form and distribution in striated muscle fibres. *J. biophys. biochem. Cytol.* **3**, 269–300.

ROBERTSON, J. D. (1956). Some features of the ultrastructure of reptilian skeletal muscle. *J. biophys. biochem. Cytol.* **2**, 369–380.

J. Physiol. (1960), **153**, *pp.* 386–403
With 12 text-figures
Printed in Great Britain

POTASSIUM CONTRACTURES IN SINGLE MUSCLE FIBRES

BY A. L. HODGKIN AND P. HOROWICZ*
From the Physiological Laboratory, University of Cambridge

(*Received* 20 *May* 1960)

It has been known for a long time that muscles can be made to contract by raising the potassium concentration in the external medium. In skeletal muscles these contractions are difficult to study, because potassium ions take some time to diffuse through the interspaces and the contractions which they set up are usually transient. The difficulties of interpretation become less acute if single fibres are employed; in that case, as was first shown by Kuffler (1946), a rise in external potassium concentration causes a rapid depolarization and a prompt contraction. The quantitative effects of sudden changes of potassium concentration on membrane potential have been described previously (Hodgkin & Horowicz, 1959, 1960); the present article deals with the effect of similar changes on the tension developed by single muscle fibres. For relevant work on whole muscles the excellent review of Sandow (1955) should be consulted.

METHODS

All the experiments were carried out with single fibres from the semitendinosus muscle of *Rana temporaria*. The apparatus and method of recording have been described in previous articles (Hodgkin & Horowicz, 1959, 1960). The only additional item of equipment was a device for changing solutions rapidly. In the method described previously, solutions were allowed to run into the cell from reservoirs at a height of about 50 cm above the cell; the flow obtained with this method was 1–3 ml./sec and the mean velocity in the channel containing the fibre was 10–30 cm/sec. In some of the present experiments (Fibres *D*, *E*, *F* in Table 1 and Figs. 1 and 2) the reservoirs were replaced by large syringes (internal diameter 2·1 cm) whose plungers were driven by pistons operated by compressed air between stops spaced 0·7 cm apart. This forced 2 ml. of solution through the cell in about 0·3 sec. Tests with dyes showed that the solution in the cell (volume about 0·3 ml.) was fully changed in one flush. The rate of flow (about 60 cm/sec) produced by the piston-operated syringes was too high for the experiments in which the membrane potential was recorded and most of the results described here were obtained with the original method.

Solutions were made up on the same lines as those described previously (Hodgkin & Horowicz, 1959, Table 1). When using relatively short exposures to high $[K]_o$, $[Cl]_o$ was generally kept at its normal concentration of 120 mM. For longer exposures solutions of constant [K] [Cl] product were employed, in order to avoid loading the fibre with Cl.

* Present address: Department of Physiology, Washington University, St Louis, Missouri, U.S.A.

RESULTS

The relation between potassium concentration and peak tension

The muscle fibres used here were of the quick, phasic type and did not give maintained contractions when depolarized with high potassium concentrations. The way in which the muscle relaxes in high $[K]_o$ will be described later; to begin with we shall consider the effect of relatively short exposures in which the contraction was cut short by a return to low $[K]_o$ and not by a spontaneous relaxation. Figure 1 shows the effect of different potassium concentrations on the tension generated by the

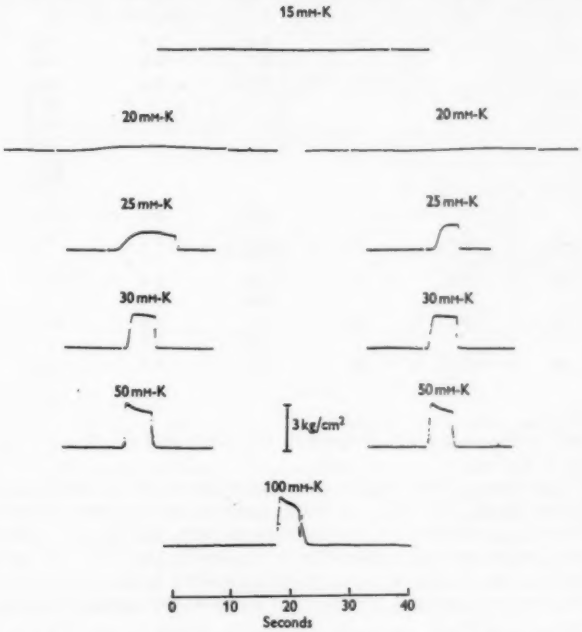


Fig. 1. Tension resulting from brief applications of potassium concentrations, varying between 15 and 100 mM-K, to fibre in choline-Ringer's fluid. Fibre *E*, diameter 88 μ, temperature 16·5° C; Na-free solutions containing [K + choline] = 120 mM, 121 mM-Cl 1·8 mM-Ca, 1·5 mM phosphate buffer. The potassium concentration at the beginning and end of each record was 2·5 mM. The breaks in the record, which were caused by the sudden flow of fluid through the cell, mark the times at which high $[K]_o$ was applied or removed. The fibre was allowed to rest for 7–10 min between contractures. The order of the records was 20–50 mM (left-hand column), 100–15 mM (bottom, right-hand and top). The fibre was stretched to 1·28 times slack length, giving a measured sarcomere distance of 2·7 μ.

fibre; solutions were made up with choline instead of sodium, so there were no action potentials or twitches when $[K]_o$ was increased. Contractions began at 20 mM-K, reached half their maximum at 25 mM-K and were nearly maximal at 50 mM-K. In this fibre the maximum tension at 100 mM-K was 3·0 kg/cm² and was 11 % greater than that produced by a 125/sec tetanus in Na-Ringer's fluid. Table 1 shows that similar values were obtained in other experiments.

TABLE 1. Maximum tension developed in tetani and contractures

Fibre reference	Fibre diameter (μ)	Sarcomere length (μ)	Temperature (°C)	Tetanus tension per cm² 50/sec	Tetanus tension per cm² 125/sec	Contracture tension per cm²	Contracture tension / Tetanus tension (125/sec)
				← kg/cm² →			
A	82	—	20	3·9	3·9	4·4	1·11
				3·7	4·0	4·4	
B	71	—	20	2·7	2·8	3·4	
						3·3	
						3·3	
						3·3	1·13
						3·4	
						3·3	
						3·3	
				2·9	3·1	3·3	
D	98	2·8	18	3·1	3·2	3·6	1·07
				3·3	3·5	—	
E	88	2·7	17	2·3	2·6	3·0	1·11
				2·6	2·8	—	
F	73	2·7	19	2·8	3·2	3·5	1·08
				3·3	3·3	—	
J	80	(2·5)	20	3·6	3·8	4·1	
						4·1	1·08
						4·1	

Contractures were induced with 100 mM-K, 120 mM-Cl in *D*, *E*, *F* and with 190 mM-K, 2–4 mM-Cl in *A*, *B*, *J*.

The sarcomere distance was measured after the experiment by stretching the fibre to the experimental length in a cell in it which could be observed with a water-immersion objective. The sarcomere length in *J* is based on the fact that this fibre was stretched to 1·20 times slack length, whereas *D*, *E* and *F* were stretched to 1·35, 1·28 and 1·28, respectively. Diameters are means of several measurements including major and minor diameters; errors in cross-section might be ± 20 % but this does not affect the final column.

The relation between tension and log $[K]_o$ is given by the circles in Fig. 2. In the lower part of the curve the tension increased extremely steeply with potassium concentration, the tension at 25 mM being about 5 times that at 20 mM.

The records in Fig. 1 show that the tension rose with a long delay along an S-shaped curve when $[K]_o$ was raised to 20 or 25 mM, but dropped abruptly when 2·5 mM-K was restored. With 100 mM-K the situation was reversed, for the tension rose rapidly but did not drop at once when 2·5 mM-K was restored. These effects are probably explained by the

combined action of a delay in the response of the membrane potential to a change in $[K]_o$ and a very steep relation between membrane potential and tension (p. 393).

There was a slight difference between the contracture thresholds in choline- and in Na-Ringer's fluid. Tension log $[K]_o$ plots indicated that

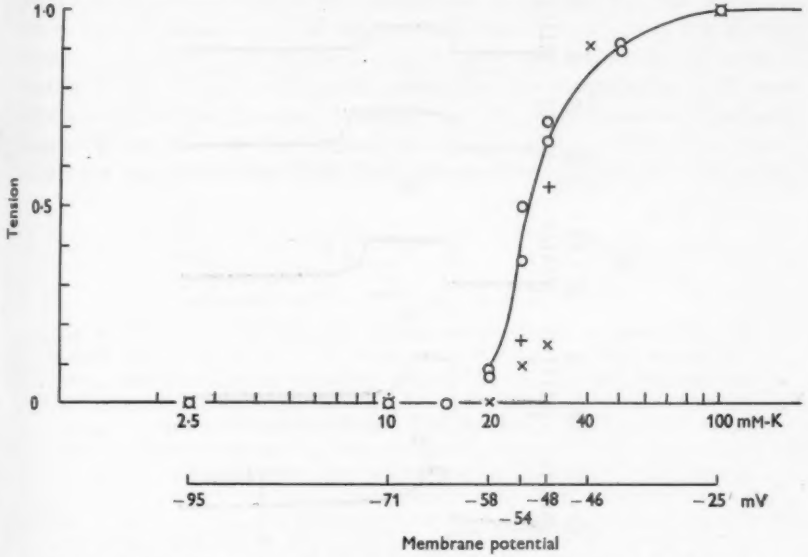


Fig. 2. Relation between peak tension and potassium concentration or membrane potential. ○, Fibre *E*, tension only. +, Fibre *G*, tension only. ×, Fibre *G*, tension and membrane potential. The numbers attached to the lower scale are the internal potentials measured at the same time as the tension. The scale for the potassium concentration is logarithmic and for potential is approximately linear, the difference in scale corresponding to 45 mV for a tenfold change. Choline-Ringer's fluid with K replacing choline. Fibre *E*, diameter 88 μ, maximum tension 3·0 kg/cm², temperature 16·5° C. Fibre *G*, diameter 75 μ, maximum tension 4·0 kg/cm², temperature 18° C.

in the presence of Na^+ the curve was shifted to the right by a factor of about 1·2. For example, 25 mM-K choline-Ringer gave roughly the same tension as 30 mM-K Na-Ringer. The effect is in the same direction, but much smaller, than that observed in heart muscle by Lüttgau & Niedergerke (1958).

The relation between membrane potential and tension

Figure 3 illustrates some simultaneous recordings of tension and membrane potential. Contractions began at a potential of about −54 mV

(25 mM-K) and were nearly maximal at −46 mV (40 mM-K). The relation between tension and membrane potential is shown by the crosses and the lower scale in Fig. 2. Figure 4 shows that a small increase in depolarization produced measurable tension and that this was suppressed by a small decrease in depolarization.

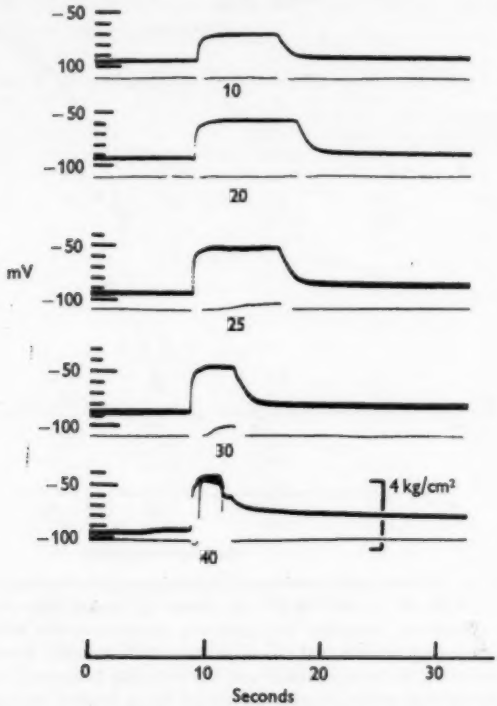


Fig. 3. Simultaneous recording of internal potential (upper trace) and tension (lower trace) showing effect of brief application of potassium concentrations varying between 10 and 40 mM to fibre in choline-Ringer's fluid containing 2·5 mM-K. The record in 30 mM-K was taken immediately after that in 25 mM-K and this may have reduced the size of the mechanical response; earlier measurements, in which the fibre was allowed to rest between contractions, gave 3·4 times more tension in 30 mM-K than in 25 mM-K. Fibre *G*, diameter 75 μ, temperature 18° C. Na-free solutions with [K]+[choline] = 120 mM, 121 mM-Cl, 1·8 mM-Ca, 1·5 mM-phosphate buffer. The tension record has been slightly retouched.

The sharpness of the contractile threshold

If the latency and slow rise of tension depend solely on the time taken by the membrane potential to pass through a critical region, a comparison of tension and potential during the rising phase of a contracture should

give the relation between the two variables; for short contractures the same relation ought to hold for the sudden relaxation associated with a reduction of $[K]_o$. These measurements could not be carried out in a direct manner because the onset of contraction was associated with irregularities in the recorded potential. Figure 5 illustrates the procedure adopted. Curve *A* gives the membrane potential for the sequence 10 → 20 → 10 mM-K, and *B* the potential for 10 → 30 → 10 mM-K; the latter resulted in a substantial rise in tension which is shown in curve *b*. Curve *B** was drawn on the assumption that the irregularities in *B* were errors and that the internal potential rose smoothly towards the steady value determined by the potassium concentration. Since the microelectrode was jerked out of the fibre at the end of the period in 30 mM-K,

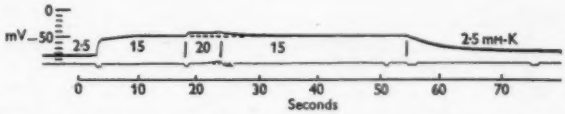


Fig. 4. Effect of sudden changes in potassium concentration on internal potential (upper trace) and tension (lower trace). Fibre *H*, diameter 119 μ, temperature 18° C, sulphate solutions containing 8 mM-$CaSO_4$ and [Na+K] = 83 mM (see Hodgkin & Horowicz, 1959, Table 1, solutions *D* and *E*). At 51 and 75 sec the solution already in the cell was flushed through for a second time. The tension developed in 20 mM-K was 4 % of the maximum contraction tension (3·2 kg/cm²). The record was taken several minutes after impalement and the resting potential in 2·5 mM-K had declined 7 mV during this period; 2 hr earlier the resting potential was 95 mV. The changes in tension and potential produced by replacing 15 with 20 mM-K are best seen by looking along the record.

the repolarization phase of *B** had to be inferred from the repolarization observed in record *A*. If the tension in the initial part of curve *b* is plotted against membrane potential, it is found that each millivolt increase in depolarization is associated with an elevenfold rise in tension. This is illustrated by the circles near the tension record, which were calculated on the assumption that tension $\propto \exp(V/0{\cdot}417)$. The same equation gave an approximate fit to the phase of relaxation at the end of the period in high $[K]_o$. Similar results were obtained in two other experiments, but the value of the exponential constant was somewhat higher, the mean in the three experiments being 0·7 mV. This figure is subject to large errors, but it is clear that the tension generated in the region of the mechanical threshold is acutely sensitive to small changes in membrane potential.

Absolute value of the contractile threshold

In five experiments with fibres equilibrated in choline-Ringer's fluid, the critical potential for development of measurable tension was found to average −54 mV (range from −48 to −58 mV). Similar measurements

were not made with ordinary Ringer's fluid because the fibres often gave a twitch when $[K]_o$ was increased; this would have damaged the membrane if the electrode had been inserted. Since about 20 % more K was required to give a contracture in Na than in choline solutions the critical potential in the presence of Na may be taken as about −50 mV. Critical potentials of from −40 to −45 mV were obtained in fibres which had been equilibrated in sulphate solutions containing 10 mM-K and 70 mM-Na; these fibres had resting potentials of −65 mV instead of the usual −95 mV.

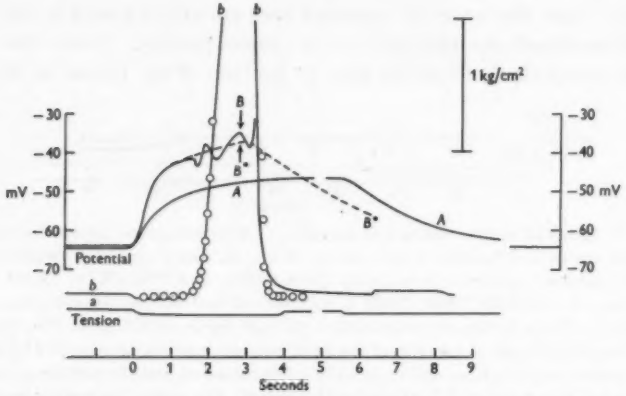


Fig. 5. Simultaneous records of membrane potential (*A*, *B*) and tension (*a*, *b*). The changes in solution were

Aa: 10 → 20 → 10 mM-K (0-Cl)
Bb: 10 → 30 → 10 mM-K (0-Cl).

In *A* 20 mM-K was applied for 20 sec and the gap in the record represents 14·5 sec. In *B* 30 mM-K was applied for 3 sec. The micro-electrode was jerked out of the fibre when relaxation started. Curve *B**, which is based on *B* and *A*, gives the assumed time course of the membrane potential. The circles against the tension curve *b* were calculated from *B** by the equation

$$T = 0{\cdot}284 \exp \frac{V+40}{0{\cdot}417},$$

where T is tension per unit area in kg/cm² and V is the internal potential in mV. Fibre *K*, diameter 117 μ, temperature 20° C, sulphate solutions as in the experiment of Fig. 3. Another record showed that the tension in 30 mM-K reached a maximum value of 3·2 kg/cm² and that with a maintained exposure to 30 mM-K the duration of the contracture was about 20 sec.

The time course of the contraction during a maintained depolarization

As is to be expected in a phasic muscle, the contraction was not maintained when the fibre was depolarized for periods longer than 5–30 sec. Figure 6 shows simultaneous recordings of membrane potential and tension

during a contraction produced by high $[K]_o$. The record starts with the fibre in a solution containing 190 mM-K and 3·6 mM-Cl; the membrane potential was close to zero. The fibre contracted when first put into high $[K]_o$ but had relaxed completely before the beginning of the record. On applying Ringer's fluid (2·5 mM-K, 120 mM-Cl) the fibre repolarized to about −90 mV. After 43 sec in Ringer's fluid, 190-K 3·6-Cl was again applied; the membrane potential fell rapidly to zero and the fibre developed a peak tension of 4·3 kg/cm². The contraction was not maintained and after a few seconds the fibre was fully relaxed, in spite of the fact that the membrane potential remained close to zero.

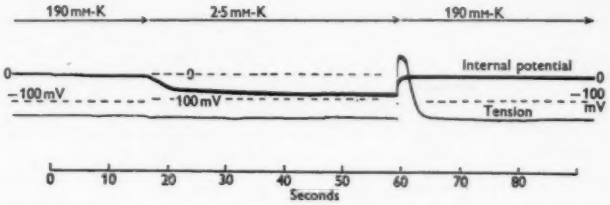


Fig. 6. Simultaneous records of membrane potential and tension for following changes in solution

190 mM-K 3·6 mM-Cl → 2·5 mM-K 120 mM-Cl → 190 mM-K 3·6 mM-Cl.

The peak tension developed was 4·3 kg/cm². In this experiment the internal potential was recorded against an agar-Ringer electrode in the external solution. The dotted lines allow for the junction potential. (In the other experiments illustrated the effect of the junction potential was small or was eliminated by recording with a 3 M-KCl electrode in the external solution.) The fibre was impaled at zero time. On reapplying Ringer's fluid after the end of the record the fibre repolarized to −75 mV, showing that the electrode had not been dislodged by the contraction. Fibre *C*, diameter 62 μ, temperature 21° C.

A characteristic feature of the contractures recorded from single muscle fibres is the rather sudden transition from a plateau, in which the tension fell slowly, to a rapid exponential phase of relaxation (Figs. 6, 7, 9 and 10). Occasionally the tension was constant during the plateau but a gradual decline was more usual. A decline in tension comparable to that in the plateau of the contracture was also seen if the fibre was stimulated at 50 or 125/sec for more than a second. This gradual decline took place before the irregular fall in tension resulting from failure of action potentials had begun.

Another interesting point about the contractures produced by high $[K]_o$ is that the duration of the plateau and the time constant of the phase of relaxation both shortened progressively as the potassium concentration was increased from 50 to 190 mM (Fig. 7).

A tentative explanation of the plateau and of the shortening of the contracture with increasing $[K]_o$ can be given in the following way. Suppose that depolarization releases an activator which is destroyed in a first-order reaction with a rate constant of about 30 sec^{-1}. A very brief depolarization of an action potential might then give a twitch lasting 30–100 msec, whereas a maintained depolarization would lead to a contraction lasting until the precursor of the activator was nearly exhausted.

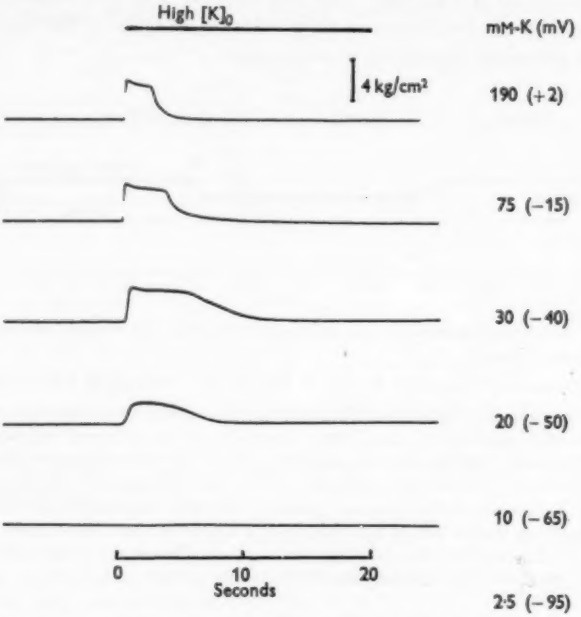


Fig. 7. Tension resulting from sudden applications of high $[K]_o$ and low $[Cl]_o$. The chloride concentration was $(300/[K]_o)$ mM, except with $[K]_o = 190$ mM where there was 2·5 mM-Cl; solutions were of the constant K, Cl product type (see Hodgkin & Horowicz, 1959, Table 1, *F* and *G*). Potentials were taken from Fig. 4 of that paper. The high-K low-Cl solutions contained some Na, but twitches were prevented by keeping the fibre in choline-Ringer's fluid (2·5 mM-K, 120 mM-Cl) before switching on the test solution. Fibre *I*, diameter 106 μ, temperature 21° C.

Since the depolarization produced by potassium concentrations greater than 50 mM is greater than the mean depolarization in a train of action potentials at a frequency of 100/sec, one would expect high potassium concentrations to raise the concentration of activator above the level required to saturate the contractile mechanism. On this basis the duration of the plateau is the time for the concentration of activator to fall from

a supramaximal to a maximal value, and the duration of the relaxation phase is the time for the concentration to fall from a maximal to a threshold value (Fig. 8). High potassium concentrations might shorten the contracture by promoting the rate of liberation of the activator from its precursor and by reducing the rate at which the precursor is resynthesized. If something like this is correct it should be possible to shorten the plateau, or to eliminate it altogether, by reducing the amount of precursor.

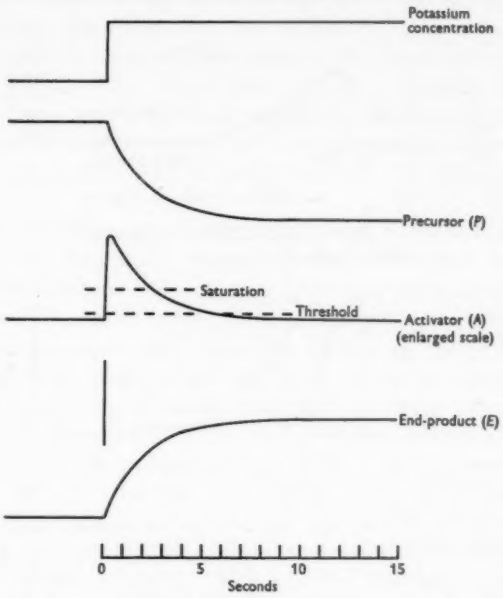


Fig. 8. Diagram showing rise and fall of hypothetical 'activator' during a potassium contracture. The curves are drawn as

$$\text{Precursor } (P) = e^{-\alpha t};$$

$$\text{Activator } (A) = \frac{\alpha}{\beta-\alpha}(e^{-\alpha t}-e^{-\beta t});$$

$$\text{End product } (E) = 1-(P+A),$$

where $\beta = 30$ sec^{-1}; $\alpha = 1$ sec^{-1}. Scale for (A) 29 times that for (P) or (E). The equations were obtained by neglecting back reactions in the system: $P \xrightarrow{\alpha} A \xrightarrow{\beta} E$.

Figure 9 illustrates an experiment suggested by this hypothesis. Curve $A1$ shows the tension produced by applying 100 mM-K to a fibre which had been equilibrated in Ringer's fluid (2·5 mM-K), whereas $A2$ shows the effect of applying 100 mM-K after the fibre had been recovering for a relatively short time (38 sec) in Ringer's fluid. It will be seen that the second contraction is smaller, that it had no plateau and that it could be

superposed on the falling phase of the first contraction. These findings agree well with the idea that relaxation depends on the exhaustion of a store of activator, but could no doubt be explained in other ways, for example, by assuming a constant level of activator and the slow development of an inhibitory process. Similar results were obtained in a series of experiments with a higher potassium concentration (190 mM), provided the first exposure to high $[K]_o$ was short (Fig. 9*B*). However, if the first exposure was long, the relaxation after the second contraction was more

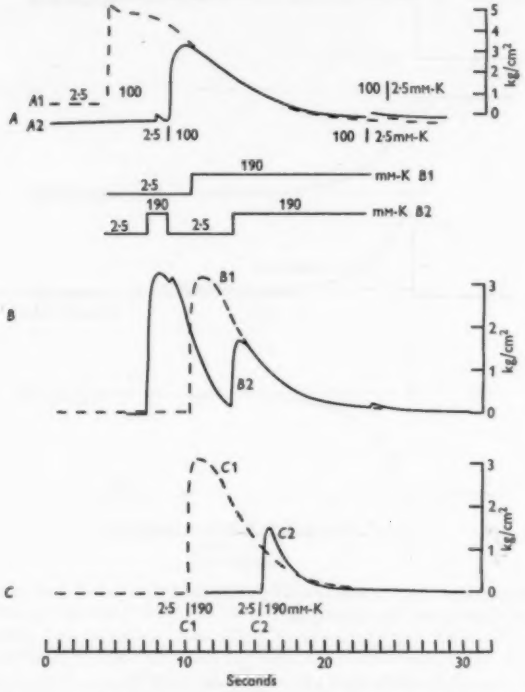


Fig. 9. Comparison of contractures produced by applying high [K] to resting fibres and to those recovering from previous exposure to high [K].

A. Fibre diameter *ca*. 50 μ, temperature 20° C. *A* 1, effect on resting fibre of replacing 2·5-K 120-Cl with 100-K 120-Cl. *A* 2, same change in solution but applied when the fibre had been recovering in 2·5-K 120-Cl for 38 sec after removing 100-K in record *A* 1.

B. Fibre diameter 71 μ, temperature 20° C. The potassium concentrations are shown on the figure. The 2·5-K solution was Ringer's fluid (120-Cl) and the 190-K was an isotonic sulphate solution containing 3·6 mM-Cl.

C. Same fibre and solutions as *B*. *C* 1, application of 190-K 3·6-Cl to resting fibre. *C* 2, application of 190-K 3·6-Cl to fibre recovering from *C* 1 (115 sec in 190-K 3·6-Cl followed by 18·5 sec in 2·5-K 120-Cl).

rapid than after the first (Fig. 9*C*) and the two curves were no longer superposable. Another example of the difference between the effects of long and short exposures to high $[K]_o$ is mentioned on p. 400.

The 'steady' relation between membrane potential and the state of the contractile system

When single fibres are immersed in high $[K]_o$ they contract and then relax completely. On returning them to low $[K]_o$ they repolarize, and after a short time (usually 5–30 sec) they give a second contraction when reimmersed in high $[K]_o$. The return of the contractile system or its activating mechanism to a resting condition will be referred to as the priming or repriming of the system and the converse process which causes relaxation in high $[K]_o$ will be said to make the fibre mechanically refractory.

The question considered here is the steady relation between the potassium concentration (or membrane potential) and the extent to which the contractile system is refractory. The experimental procedure is illustrated by Fig. 10. The contractures on the left were obtained by applying 190 mM-K, 2·5 mM-Cl after the fibre had rested for 10 min in Ringer's fluid. Immediately after these contractions the fibre was immersed for 1 min in a test solution containing x mM-K and $(300/x)$ mM-Cl (see Hodgkin & Horowicz, 1959). After 1 min in the test solution 190 mM-K was applied for a second time. If the potassium concentration in the test solution was low the fibre contracted and the size of the second contracture was taken as an index of the amount of restoration. The records showed that there was no restoration in 40 mM-K, a little in 30 mM-K and practically complete recovery in 20, 10 or 2·5 mM-K. The reason for choosing an equilibration time of 1 min in the test solution was that recovery in 2·5 mM-K was very nearly complete in that period and that with a solution such as 30 mM-K the restoration reached a maximum at about 1 min and then underwent a slow decline. The decline may perhaps be a result of the very large rise in oxidative heat production which occurs when muscles are immersed in solutions containing 20–30 mM-K (Hill & Howarth, 1957).

Figure 11 gives the relation between the potassium concentration and the state of the contractile system. Probable values of the membrane potential are given by the lower abscissa. The filled circles are the height of the second contraction and the crosses the areas under the second contraction. The latter are considered to be a better index of the extent to which the contractile system has been restored. It will be seen that curve 3, which runs through the crosses, is a mirror image of the relation between membrane potential and initial tension (open circles, curve 1).

The initial-tension curve was obtained in the usual way by starting with the fibre in 2·5 mM-K, switching on x mM-K and measuring the height of the contraction in this solution.

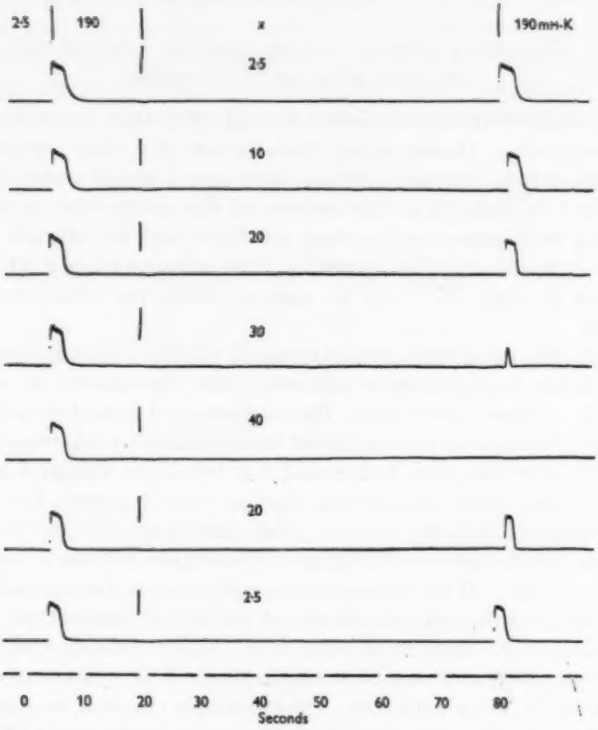


Fig. 10. Effect of allowing fibre to recover for 1 min in solution containing x mM-K and $(300/x)$ mM-Cl on tension resulting from second application of 190 mM-K. The sequence of solutions was: 2·5-K 120-Cl; 190-K 2·5-Cl; x-K $(300/x)$-Cl; 190-K 2·5-Cl. All records were at the same gain; the peak tension in the first contracture was 4·1 kg/cm². Fibre J, diameter 80 μ, temperature 20° C.

Recovery of twitches and repriming of the contractile system after a period in high $[K]_o$

Figure 12, record A, shows the effect of applying 190-K 3·6-Cl for 60 sec during a period when the fibre was being stimulated steadily at 0·78 shocks/sec. The twitches were abolished by the high-K solution and did not reappear until 11 sec after restoring Ringer's fluid. The recovery time varied with the duration of the exposure to high K; times as short as 6 sec were observed after exposures of 3 sec, but if 190 mM-K was applied

for 3–10 min the recovery time might increase to about 1 min. The recovery time also appeared to grow progressively longer as the fibre deteriorated. As one would expect, the recovery was quicker if concentrations lower than 190 mM-K were used to depolarize the fibre.

It was interesting to know if there was any relation between the recovery of twitch amplitude and the repriming of the contractile system. This could be done simply by reapplying 190 mM-K during the period when the fibre was recovering in 2·5 mM-K.

Record *B* in Fig. 12 shows that when 190 mM-K was reapplied at a time when the twitch had nearly recovered it gave full tension but a contracture which was shorter than normal. Record *C* shows that when 190 mM-K was reapplied before the twitches had appeared it gave no contracture and

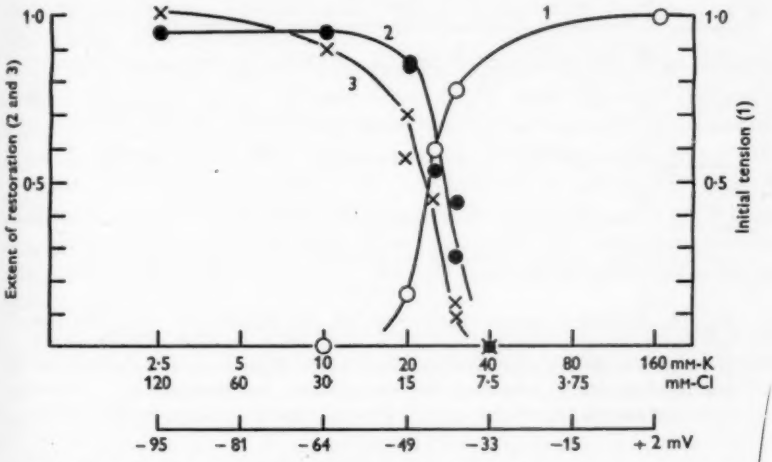


Fig. 11. Relation between potassium concentration and initial tension (curve 1) or degree of restoration (curves 2 and 3).

The abscissa gives the potassium or chloride concentrations of the test solution on a logarithmic scale. The lower scale, which is approximately linear, gives probable values of membrane potential, taken from Fig. 4 of Hodgkin & Horowicz (1959). A correction for activity coefficient has been made in plotting the right-hand point (190 mM-K 2·5 mM-Cl) on the potassium scale.

For curve 1 (○) the ordinate is the initial tension on increasing K from 2·5 to x mM, relative to the maximum tension of 4 kg/cm². (Except for the right-hand point [K] [Cl] = 300 (mM)² throughout.)

For curve 2 (●) the ordinate is the tension in 190 mM-K 2·5 mM-Cl after 1 min recovery in x mM-K $(300/x)$ mM-Cl.

For curve 3 (×) the ordinate is the area under the tension–time curve for the same contractures as in curve 2. The ordinates in curves 2 or 3 are given relative to the peak tension or area in the first contraction. Experimental details as in Fig. 10.

that when the twitch was small the contracture was small and brief. The conclusion is that the ability to give both twitches and contractures depends on the same underlying process.

One possible explanation of the simultaneous recovery of twitches and contractures is that the action potential recovers quickly and that both twitches and contractures are limited by the disappearance of the state of mechanical refractoriness. If this were so, there should be a large spike at a time when the fibre is still unable to contract. The expectation was not fulfilled, for all fibres tested were found to remain electrically inexcitable as long as they were mechanically quiescent. The first electrical

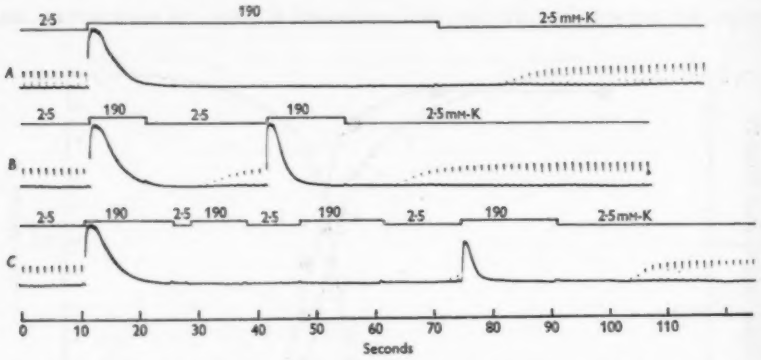


Fig. 12. Records of tension during twitches and contractures. The fibre was stimulated throughout at 0·78/sec with a shock which was well above threshold in Ringer's fluid. The external solution was either Ringer's fluid (2·5 mM-K, 120 mM-Cl) or 190 mM-K, 3·6 mM-Cl. All records at the same gain; the peak tension in the first contracture was 3·2 kg/cm². Fibre *B*, diameter 71 μ, temperature 20° C.

responses, which were greatly reduced in amplitude and probably decremental, appeared at the same time as the first twitches and both then grew in amplitude. The delay in recovery of the spike was partly due to the slow return of the resting potential (Hodgkin & Horowicz, 1960) but this was probably not the full explanation. After being kept 10 min in 190-K 3·6-Cl two fibres remained inexcitable for about 1 min in 2·5-K 120-Cl, although a resting potential of 70–80 mV was established in 20–30 sec. Apparently exposure to high $[K]_o$ has some slow effect which can persist for a minute or two and which impairs both the electrical and the mechanical response of the fibre. It is unfortunate that shortage of time and the difficulty of the experiments prevented us from making any proper investigation of this phenomenon.

DISCUSSION

The size and rapid onset of the maximal potassium contractures in single muscle fibres strongly supports the conclusion of Kuffler (1946), Sten-Knudsen (1954) and Sandow (1955) that the event which normally induces contraction is a change of membrane potential rather than a longitudinal current. With the present method there might be small longitudinal currents during the fraction of a second required to change solutions, but at longer times any longitudinal currents should be exceedingly small compared with those in the propagated impulse. Nevertheless, the tension generated with high $[K]_o$ in an Na-free solution can remain for several seconds at about the same level as that in a maximal tetanus. Since the fibre relaxes when $[K]_o$ is reduced and since a maximal contraction can be repeated many times, it cannot be argued that any irreversible effect is involved.

The observation that the maximum contracture tension is about 10% greater than the maximal tetanic tension is new but not altogether surprising. At 19° C the mean depolarization associated with a 125/sec train of spikes is 40–50 mV as against the steady depolarization of 60–100 mV produced by 100–190 mM-K (unpublished records and Hodgkin & Horowicz, 1959). A quantitative comparison cannot be made until more is known about mechanical activation, but it does not seem unreasonable that the steady depolarization should be more effective, in spite of the higher peak reached in the train of spikes.

There seems to be general agreement that in normal muscle fibres the strength of current, or the degree of depolarization, required to activate the mechanical system is not much greater than the threshold for the propagation of excitation (Ramsey & Street, 1938; Kuffler, 1946; Taylor, 1953). Since the action potential normally arises at a potential of -45 to -60 mV (Fatt & Katz, 1951; Jenerick & Gerard, 1953) our value of about -50 mV for the contractile threshold is not unreasonable. Although it was difficult to obtain quantitative results, our experiments show that there is an extremely steep relation between tension and membrane potential in the region of the mechanical threshold. A sharp threshold might be expected if depolarization started an autocatalytic or regenerative reaction. However, there was evidence against any regenerative process, for contractures were never 'all-or-nothing' and the tension varied continuously and reversibly with membrane potential. A possible explanation of the steep relation is that there is no appreciable tension until one reaction initiated by depolarization overtakes another which has the opposite effect. (Something of this kind seems needed to explain latency relaxation.) Alternatively it might be supposed that the fibre does not

develop measurable tension until many sites have been activated. An experiment which ought to be done is to see whether any motion, detectable with a high-power microscope, occurs at potassium concentrations which are just below the mechanical threshold.

In the second part of the paper it is shown that contractures produced by potassium concentrations greater than 50 mM consist of an initial plateau and a second phase in which the tension falls in an approximately exponential manner to its resting value. The durations of the plateau and the time constant of the relaxation shorten progressively as the depolarization is increased. It is suggested that relaxation might be caused by the exhaustion of an activator, or by the accumulation of something produced by the activator, and that the brevity of the contractures in high $[K]_o$ may be due to release of activator at a rate which is wastefully high.

The experiments show that a fibre which has relaxed in high $[K]_o$ can be restored to a condition in which it is once more able to contract by reducing the potassium concentration to less than 30 mM. Restoration of the contractile system usually occurs in under a minute and in the steady state the degree of restoration is related to membrane potential by an S-shaped curve which is roughly the mirror image of the curve relating initial tension to membrane potential. A tentative hypothesis is that reduction of the normal potential difference across the membrane liberates an activator which is used up in generating tension, whereas an increase in membrane potential prevents the activator being destroyed and allows its concentration in a store to be built up to a high level.

Our experiments give no information about the localization of the hypothetical activator, but from the work of Huxley & Taylor (1958) it is conceivable that the electrical effect of the normal resting potential might be to concentrate a negatively charged particle in some part of the endoplasmic reticulum (Porter & Palade, 1957; Huxley, 1959). On this basis depolarization would start a contraction by allowing an activating particle —possibly a negatively charged Ca complex—to move into the main part of the muscle, and the duration of the contraction might depend on the time taken to exhaust the activator stored in the reticulum.

SUMMARY

1. A sudden increase of the external potassium concentration, $[K]_o$, from 2·5 to 100 mM-K caused a single muscle fibre to develop a tension of 2–4 kg/cm^2 in a fraction of a second.

2. The maximum contracture tension was about 1·1 times the maximum tetanus tension.

3. Development of tension started at 20–30 mM-K and was related to log $[K]_o$ or membrane potential by a steep S-shaped curve.

4. The membrane potential at which contractions started was usually about -50 mV.

5. The relation between membrane potential and tension was reversible and, with fibres in Na-free solutions, there was no evidence of a regenerative process.

6. When $[K]_o$ was maintained at a high level the tension declined to its resting value along a characteristic curve consisting of an initial plateau and a subsequent phase of rapid relaxation.

7. The duration of the plateau and the time constant of relaxation shortened progressively as the potassium concentration was increased.

8. After a fibre had relaxed in high $[K]_o$ it could be restored to a condition in which it was once more able to contract by reducing $[K]_o$ for 10–30 sec below 20–30 mM. The degree of restoration was related to log $[K]_o$ (or membrane potential) by an S-shaped curve with a half-value at 20–25 mM-K (or -50 to -45 mV).

9. When Ringer's fluid was used to restore a fibre which had relaxed in high $[K]_o$, the action potential, twitches and the ability to give contractures all returned at about the same time.

REFERENCES

FATT, P. & KATZ, B. (1951). An analysis of the end-plate potential recorded with an intracellular electrode. *J. Physiol.* **115**, 320–370.

HILL, A. V. & HOWARTH, J. V. (1957). The effect of potassium on the resting metabolism of the frog's sartorius. *Proc. Roy. Soc.* B, **147**, 21–43.

HODGKIN, A. L. & HOROWICZ, P. (1959). The influence of potassium and chloride ions on the membrane potential of single muscle fibres. *J. Physiol.* **148**, 127–160.

HODGKIN, A. L. & HOROWICZ, P. (1960). The effect of sudden changes in ionic concentrations on the membrane potential of single muscle fibres. *J. Physiol.* **153**, 370–385.

HUXLEY, A. F. (1959). Local activation of muscle. *Ann. N.Y. Acad. Sci.* **81**, 446–452.

HUXLEY, A. F. & TAYLOR, R. E. (1958). Local activation of striated muscle fibres. *J. Physiol.* **144**, 426–441.

JENERICK, H. P. & GERARD, R. W. (1953). Membrane potential and threshold of single muscle fibres. *J. cell. comp. Physiol.* **42**, 79–102.

KUFFLER, S. W. (1946). The relation of electrical potential changes to contracture in skeletal muscle. *J. Neurophysiol.* **9**, 367–377.

LÜTTGAU, H. C. & NIEDERGERKE, R. (1958). The antagonism between Ca and Na ions on the frog's heart. *J. Physiol.* **143**, 486–505.

PORTER, K. R. & PALADE, G. E. (1957). Studies on the endoplasmic reticulum. III. Its form and distribution in striated muscle fibres. *J. biophys. biochem. Cytol.* **3**, 269–300.

RAMSEY, R. W. & STREET, S. F. (1938). The alpha excitability of the local and propagated mechanical response in isolated single muscle fibres. *J. cell. comp. Physiol.* **12**, 361–378.

SANDOW, A. (1955). Contracture response of skeletal muscle. *Amer. J. physical Medicine*, **34**, 145–160.

STEN-KNUDSEN, O. (1954). The ineffectiveness of the 'window field' in the initiation of muscle contraction. *J. Physiol.* **125**, 396–404.

TAYLOR, R. E. (1953). The contractile process is not associated with potential changes. *J. cell. comp. Physiol.* **42**, 103–123.

J. Physiol. (1960), **153**, *pp.* 404–412
With 5 text-figures
Printed in Great Britain

THE EFFECT OF NITRATE AND OTHER ANIONS ON THE MECHANICAL RESPONSE OF SINGLE MUSCLE FIBRES

By A. L. HODGKIN and P. HOROWICZ*
From the Physiological Laboratory, University of Cambridge

(*Received* 20 *May* 1960)

This paper is concerned with two experiments suggested by the work of Kahn & Sandow (1950) and Hill & Macpherson (1954) on the effect of nitrate in prolonging and increasing the twitch of skeletal muscle. Both sets of authors concluded that nitrate acted rapidly and that the onset of the effect in whole muscle was probably limited by the rate at which anions could diffuse through the interspaces. The experiments described here, which were carried out with single fibres, support this conclusion by showing that the increase in twitch amplitude occurs within a few seconds of replacing chloride by nitrate. However, since the effect of nitrate was slower than the effect of sodium on excitability, the slight delay observed with nitrate cannot be attributed to the time taken to change the ionic concentrations immediately outside the fibre.

A possible explanation of the nitrate effect is that this anion reduces the depolarization needed to activate the contractile mechanism. If this is correct, the potassium concentration required to give a contracture should be reduced by nitrate. Kahn & Sandow (1955), who evidently had this idea in mind, showed that substituting nitrate for chloride increased and prolonged the contractures produced by 100 mM-K, but they did not describe the effect of nitrate on the mechanical threshold. The experiments reported here show that replacement of chloride with nitrate reduces the potassium concentration required to produce a given tension by a factor of 2–2·5.

METHODS

The experiments were carried out with single fibres from the semitendinosus muscles of *Rana temporaria*, using the method described by Hodgkin & Horowicz (1959). Except when recording membrane potentials, solutions were driven through the cell by syringes operated with compressed air (Hodgkin & Horowicz, 1960).

Ringer's fluid had the composition given under solution *A*, Table 1, of Hodgkin & Horowicz (1959). Br, NO_3, I or SCN were substituted for Cl; K was substituted for Na or choline.

* Present address: Department of Physiology, Washington University, St Louis, Missouri, U.S.A.

RESULTS

The effect of nitrate and other anions on the twitch of single fibres

The experiments to be considered first were designed to test the method of changing solutions and to see whether sodium ions had rapid access to the excitable membrane. Record *A* in Fig. 1, which illustrates the effect of flushing through the solution already in the cell, shows that the sudden flow of solution caused a slight reduction in the next twitch but that the

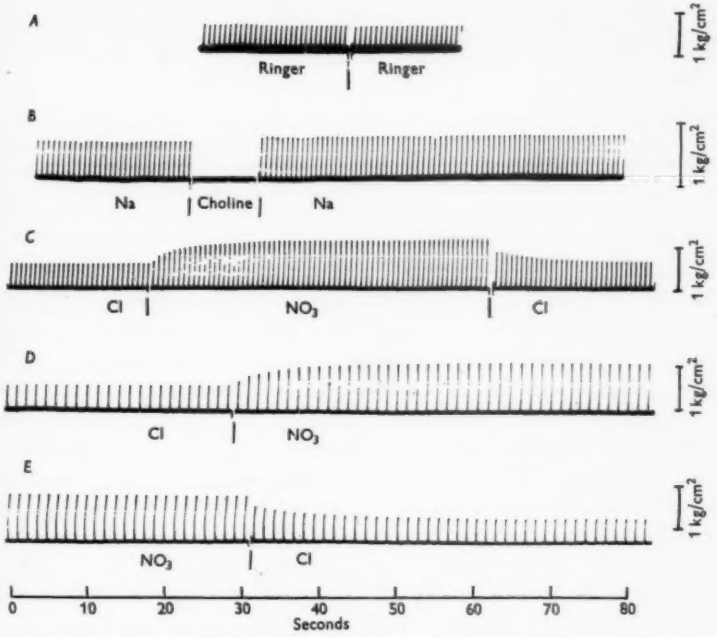


Fig. 1. Effect of sudden changes in solution on twitch tension. The solutions were changed by forcing 2·0 ml. through the cell in about 0·4 sec; the gaps in the record were caused by the rapid flow.

A, Effect of control flush in which the solution already in the cell (Ringer's fluid) was replaced with the same solution; stimulation frequency 1·56/sec.

B, Effect of replacing Ringer's fluid containing 120 mM-Na with similar solution containing 115 mM choline and 5 mM-Na; frequency 1·56/sec.

C, Effect of replacing Ringer's fluid containing 121 mM-Cl with similar solution containing 117·5 mM-NO_3 and 3·6 mM-Cl; stimulation frequency 1·56/sec.

D, *E*, Consecutive records similar to *C* but with stimulation frequency of 0·78/sec.

The records were taken in the order *C*, *D*, *E*, *B*, *A*; shortly before *C* the tetanus tension was 2·9 kg/cm². Fibre diameter 122 μ; fibre stretched to 1·18 times slack length; temperature 19° C.

reduction was not large enough to obscure the time course of the nitrate effect.

Record *B* in Fig. 1 shows the effect of an 8 sec application of a solution containing 115 mM choline and 5 mM-Na instead of Ringer's fluid with 120 mM-Na. Since the train of twitches was interrupted abruptly when Na was removed and recovered at once when Na was restored it is clear that the solution was changed quickly and that sodium ions had rapid access to the membrane. A rough estimate based on measurements of the blocking time in 5 mM-Na (about 0·6 sec) and of the concentration of Na needed to maintain the action potential (about 20 mM) is that external sodium ions disappeared with an exponential time constant of less than 0·3 sec.

While investigating the action of Na-deficient Ringer's fluid containing choline, we came across a curious effect for which there is no obvious explanation. As is clear from Fig. 1, Record *B*, the twitch recovered abruptly when Na was restored. However, if the fibre was subjected to a brief tetanus immediately after the period in choline-Ringer the tetanus tension was less than half its normal value and a full recovery did not occur until 5–10 sec after Na had been replaced. Apparently replacement of Na with choline has some additional effect which takes longer to wear off than its action on excitability.

Records *C*, *D* and *E* in Fig. 1 illustrate the action of NO_3 on a train of twitches; in *C* the fibre was stimulated at 1·56/sec and in *D* and *E* at 0·78/sec. In this fibre, which had a diameter of 122 μ, the exponential time constant was 3–4 sec for the on-effect ($Cl \rightarrow NO_3$) and 2–3 sec for the off-effect ($NO_3 \rightarrow Cl$). The mean time constant in five fibres, which had a mean diameter of 94 μ, was about 2 sec for the on-effect and about 1·5 sec for the off-effect. The time constant sometimes varied during the experiment and was shortened by stimulation at 1·56/sec for several minutes. If allowance was made for the transient reduction in twitch amplitude caused by the sudden flow of solution (the reduction was sometimes greater than in Fig. 1*A*) both the on-effect and the off-effect deviated from exponentials in the sense that the initial part of the curve was too steep. Another complication was that the twitch sometimes increased progressively in nitrate and when chloride was replaced it did not return to its former amplitude unless the fibre was rested.

Confirming previous workers (see Kahn & Sandow, 1950) we found that the potentiating effect of the anions investigated followed the sequence $Cl < Br < NO_3 < I < SCN$. Again confirming a previous suggestion (Hill & Macpherson, 1954), high concentrations of iodide and more particularly of thiocyanate were found to cause an increase in twitch amplitude which was only partially reversible. The time constant with which the twitch height increased appeared to be about the same for all the anions, although irreversible effects made it difficult to estimate the time constant

when high concentrations of I or SCN were employed. A solution containing 12 mM-SCN and 108 mM-Cl gave about the same increase in twitch height as one containing 120 mM-NO_3.

Effect of NO_3 and SCN on potassium contractures

The most obvious effect of nitrate on contractures was that it reduced the concentration at which potassium caused the muscle fibre to contract. This is illustrated by Fig. 2. Record *A* shows the effect of increasing $[K]_o$ at constant $[Cl]_o$ from 2·5 to 20 mM-K for 20 sec; there was no appreciable contraction. In record *C* a concentration of 20 mM-K again gave no increase

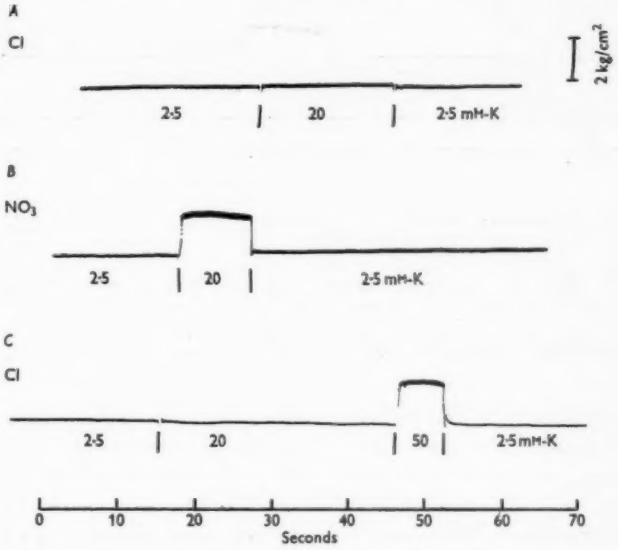


Fig. 2. Effect of replacing chloride with nitrate on tension developed at different potassium concentrations. The chloride solutions contained 121 mM-Cl, the nitrate solutions containing 117·5 mM-NO_3 and 3·6 mM-Cl. Potassium concentrations are shown on the records; [Na]+[K] = 120 mM throughout. Fibre diameter 77 μ, sarcomere length 3·2 μ, temperature 18° C. The scale applies to all records.

in tension, but on raising the concentration to 50 mM the fibre rapidly developed a tension equal to 2·0 kg/cm². In this fibre, which had been set up with a sarcomere length of 3·2 μ, the maximum tetanus tension was also about 2 kg/cm². If the potassium concentration had been kept at 50 mM the fibre would probably have remained contracted for 20–40 sec before relaxing spontaneously. Such prolonged contractions often damage the fibre irreversibly and should be avoided if possible. The contraction

was therefore cut short after 6 sec by lowering $[K]_o$ from 50 to 2·5 mM. This led to a prompt relaxation. Record *B* shows the effect of applying 20 mM-K in the presence of nitrate instead of chloride. Under these conditions 20 mM-K gave a maximal contraction which resembled that produced by 50 mM-K in the presence of 120 mM-Cl.

Figure 3 illustrates the effect of NO_3 in a different way. Starting with the fibre in chloride-Ringer's fluid, $[K]_o$ was first raised to 20 mM. This caused no contraction but on replacing Cl with NO_3 without any change in K the fibre developed substantial tension. In these experiments the tension rose and fell with a delay of 1–2 sec; this seems consistent with

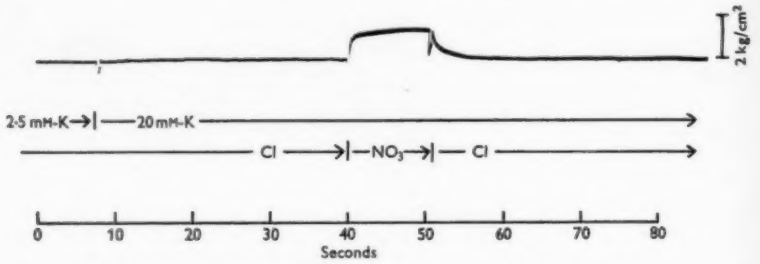


Fig. 3. Effect on tension of changing from chloride to nitrate at a constant potassium concentration of 20 mM. Same fibre and solutions as in Fig. 2. In other experiments of this kind the peak tension immediately after the change $NO_3 \rightarrow Cl$ often exceeded the steady tension in NO_3.

the conclusion from the twitch experiments that nitrate does not act instantaneously. Further evidence was provided by the observation that the change 2·5 mM-K 120 mM-NO_3 → 20 mM-K 120 mM-Cl produced a transient rise in tension, although 2·5 mM-K 120 mM-Cl → 20 mM-K 120 mM-Cl did not. Presumably the nitrate effect lasted long enough for it to cause tension when 20 mM-K 120 mM-Cl was applied.

Records similar to those in Fig. 3 were obtained in experiments with 12 mM-SCN 108 mM-Cl instead of 120 mM-NO_3. In one of these experiments all the Na was replaced by choline. Since the result was the same it is clear that the action of SCN did not depend on a change in electrical threshold or on the presence of Na in the external medium.

The effects of replacing nearly all the chloride with nitrate, or 10% of it with thiocyanate, are illustrated by Fig. 4 (NO_3) and Fig. 5 (SCN). The results are roughly fitted by assuming that the foreign anion increases the effectiveness of potassium by a factor of 2·7 (NO_3) or 2·1 (0·1–SCN). The apparently greater effectiveness of NO_3 may be misleading, since the fibre of Fig. 4 had a higher potassium threshold in chloride than the fibre

of Fig. 5. Incomplete data on another fibre which had a potassium threshold similar to that in Fig. 5 indicated that nitrate increased the effectiveness of potassium by a factor of 2·0.

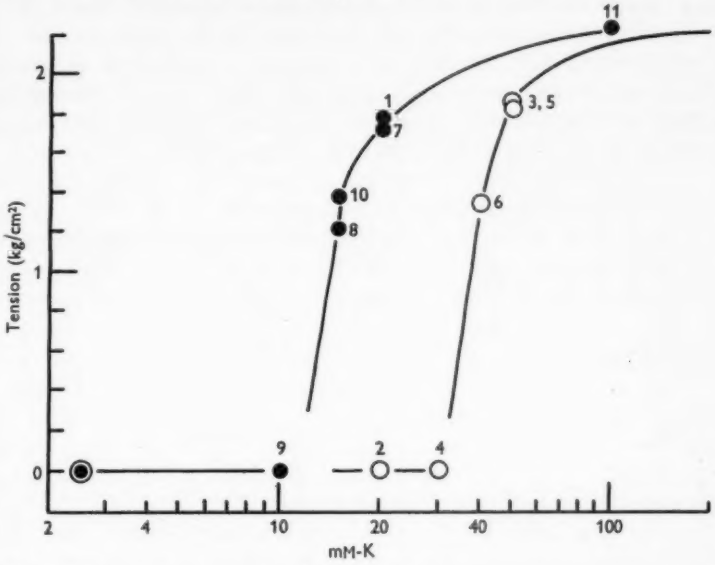


Fig. 4. Effect of nitrate on tension induced by different potassium concentrations. ○, 121 mM-Cl; ●, 117·5 mM-NO_3, 3·6 mM-Cl. Same fibre and solutions as in Fig. 2. The numbers show the order in which the measurements were made; the fibre was allowed to rest in Ringer's fluid for 10–20 min between exposures to high [K]. Semi-log scale.

Electrical measurements

The action of nitrate on the threshold concentration of potassium might arise from a shift in the mechanical threshold or from an alteration in the depolarization produced by potassium ions. One way of deciding between the two possibilities is to measure the membrane potential during experiments of the kind illustrated in Figs. 2 and 3. This was not easy. Contraction of the fibre frequently dislodged the electrode, or, if the electrode remained in position, the fibre was often damaged irreversibly. However, from the few successful experiments which were carried out it appeared that the difference in membrane potential between 20 mM-K 120 mM-Cl and 20 mM-K 120 mM-NO_3 was not more than a few millivolts. In some experiments the resting potential appeared slightly higher in nitrate, but this may have been an artifact since the resistance of the electrode often increased during a contraction and there may have been a change in tip

potential. If a change in membrane potential is ignored the shift in the tension–log $[K]_o$ curve indicates that replacing chloride with nitrate moves the tension–membrane-potential curve about 15 mV towards the resting potential. Tentative figures for the mechanical threshold are −50 mV in chloride and −65 mV in nitrate. From measurements with applied currents, Hutter & Padsha (1959) concluded that the mechanical threshold in nitrate was −65 to −75 mV; they did not measure the threshold in chloride but indicated that it was near the electrical threshold and therefore probably about −55 mV.

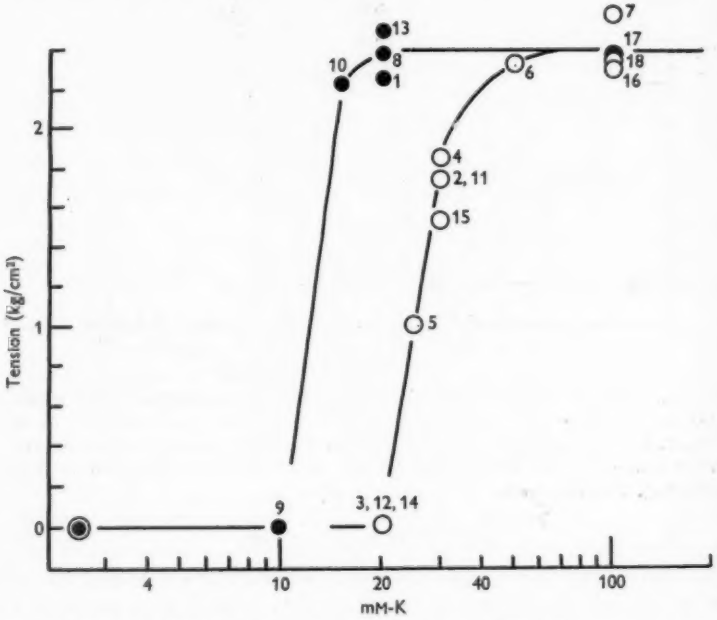


Fig. 5. Effect of 12 mM-SCN on tension induced by different potassium concentrations. ○, 121 mM-Cl; ●, 12 mM-SCN 109 mM-Cl. Fibre diameter 92 μ, sarcomere length 3·0 μ, temperature 17° C. Semi-log scale.

The absence of any appreciable change in membrane potential on switching from 20 mM-K 120 mM-Cl to 20 mM-K 120 mM-NO_3 is rather surprising. Since the nitrate conductance of the membrane is lower than the chloride conductance (Hutter & Padsha, 1959) potassium ions should cause a slightly larger depolarization in nitrate. An effect of this kind was seen when a fibre which had been in 100 mM-K 120 mM-Cl for a minute was treated with 100 mM-K 120 mM-NO_3. Under these conditions replacing chloride with nitrate caused a reversible depolarization from −25 to −18 mV. These measurements were made shortly after the fibre had relaxed and the absence of a similar change at 20 mM-K may have something to do with the fact that the fibre was contracted in nitrate but relaxed in chloride.

DISCUSSION

The main results to be considered are that anions like NO_3 or SCN caused a rapid but not instantaneous increase in twitch amplitude and that they reduced the concentration of potassium needed to give a contracture. The delay of 1–3 sec in the effect of anions on a series of twitches was several times longer than that expected from the blocking action of Na-free solutions and could not easily be attributed to diffusion through an unstirred layer outside the fibre. The alteration in contracture threshold appeared to depend mainly on a shift towards the resting potential of the curve relating tension to membrane potential.

Since the order in which the anions act (CNS > I > NO_3 > Br > Cl) corresponds to their adsorbability (Sollner, 1949, p. 1221) it is attractive to suppose that the alteration of electric field which might result from adsorption is responsible for the shift in the relation between tension and membrane potential. Some years ago, Mr A. F. Huxley suggested that calcium ions might raise the electrical threshold by adsorption at the outer edge of the membrane (see Frankenhaeuser & Hodgkin, 1956). Adsorption of an external anion should have the opposite effect and might reduce both electrical and mechanical thresholds. The experiments of Hutter & Padsha (1959) indicate that both thresholds are reduced by nitrate, but that the effect on the mechanical threshold is greater than that on the electrical threshold. This is not necessarily an objection, since the parts of the membrane concerned with propagation and mechanical activation might be spatially separated and might have different affinities for anions. Thus the sites responsible for the action potential are likely to be on the surface, whereas those concerned with activating the contractile system may be located in a tubular component of the endoplasmic reticulum (see Huxley & Taylor, 1958). Although this last suggestion is highly speculative it does have the advantage of explaining why the effects of NO_3 on twitch amplitude are slower than those of Na on propagation.

Hutter & Noble (1960) suggested that the effect of foreign anions on the twitch may be partly due to their action in prolonging the spike and after-potential and in increasing the amplitude of the after-potential. Our experiments suggest that an additional factor, which is likely to be at least as important, is the shift towards the resting potential of the relation between tension and membrane potential. On this basis the potentiating effect of foreign anions on the twitch could be attributed partly to increased activation during the spike and partly to a reduction of the mechanical threshold below the level of the after-potential.

Hill & Howarth (1957) showed that a rise in potassium concentration

caused a large increase—up to 30 times—in oxidative heat production. With Cl-Ringer's fluid the effect started at about 10 mM-K and reached a maximum at 20-mM-K; with NO_3- or I-Ringer, K was effective at 50–70% of the concentrations needed in solutions containing chloride. There is thus a striking but not an exact parallel between the effects of increasing K on the contractile system and on the rate of heat production. In both cases foreign anions augment the effect of K, but the concentrations at which K first has a detectable effect are higher for the mechanical than for the metabolic response. As Hill & Howarth remarked, it seems that a depolarization somewhat less than that required to induce contraction can release some of the chemical reactions that are normally set in motion by excitation.

SUMMARY

1. In confirmation of previous work on whole muscle, the potentiating effect of anions on the twitch of single muscle fibres was Cl < Br < NO_3 < I < SCN.

2. The effect of the anions on twitch amplitude took place with a delay of 1–3 sec.

3. 120 mM-NO_3 or 12 mM-SCN reduced the potassium concentration required to induce a given tension by a factor of about 2.

During the period of the investigation one of the authors (P.H.) held a National Science Foundation Fellowship and a National Research Council Fellowship for which funds were provided by the Rockefeller Foundation.

The expenses of the work were met by grants from the Rockefeller and Nuffield Foundations to whom we wish to express our thanks.

REFERENCES

Frankenhaeuser, B. & Hodgkin, A. L. (1956). The action of calcium on the electrical properties of squid axons. *J. Physiol.* **137**, 218–244.

Hill, A. V. & Howarth, J. V. (1957). The effect of potassium on the resting metabolism of the frog's sartorius. *Proc. Roy. Soc.* B, **147**, 21–43.

Hill, A. V. & Macpherson, L. (1954). The effect of nitrate, iodide and bromide on the duration of the active state in skeletal muscle. *Proc. Roy. Soc.* B, **143**, 81–102.

Hodgkin, A. L. & Horowicz, P. (1959). The influence of potassium and chloride ions on the membrane potential of single muscle fibres. *J. Physiol.* **148**, 127–160.

Hodgkin, A. L. & Horowicz, P. (1960). Potassium contractures in single muscle fibres. *J. Physiol.* **153**, 386–403.

Hutter, O. F. & Padsha, S. M. (1959). Effect of nitrate and other anions on the membrane resistance of frog skeletal muscle. *J. Physiol.* **146**, 117–132.

Hutter, O. F. & Noble, D. (1960). The chloride conductance of frog skeletal muscle. *J. Physiol.* **151**, 89–102.

Huxley, A. F. & Taylor, R. E. (1958). Local activation of striated muscle fibres. *J. Physiol.* **144**, 426–441.

Kahn, A. J. & Sandow, A. (1950). The potentiation of muscular contraction by the nitrate ion. *Science*, **112**, 647–649.

Kahn, A. J. & Sandow, A. (1955). Effects of bromide, nitrate and iodide on responses of skeletal muscle. *Ann. N.Y. Acad. Sci.* **62**, 137–176.

Sollner, K. (1949). The origin of bi-ionic potentials across porous membranes of high ionic selectivity I. *J. phys. Chem.* **53**, 1211–1226.